AMP-activated protein kinase regulates thyroid hormone-stimulated osteocalcin synthesis in osteoblasts

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Abstract. AMP-activated protein kinase (AMPK) is recognized as a main regulator of energy homeostasis. Osteocalcin (OC), which is produced specifically by mature osteoblasts, is stored in bone matrix, strongly binds to hydroxyapatite and is released into the circulation, has been recognized as a marker of bone metabolism. It has recently been shown that OC released from osteoblasts influences energy metabolism as a hormone. We previously reported that triiodothyronine (T_3) stimulates the synthesis of OC in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether AMPK participates in T_3-stimulated OC synthesis in osteoblasts. T_3 time-dependently induced the phosphorylation of the AMPKα-subunit (Thr-172), whereas T_3 failed to induce the phosphorylation of AMPKα-subunit (Ser-485), AMPKβ-subunit (Ser-108) and AMPKβ-subunit (Ser-182). Both the release and the mRNA expression of OC induced by T_3 were significantly inhibited by compound C, an AMPK inhibitor. Compound C suppressed the T_3-induced phosphorylation of acetyl-CoA carboxylase, a direct substrate of AMPK. T_3-stimulated OC release was significantly reduced in AMPK-knockdown cells using AMPK-siRNA. These results strongly suggest that AMPK positively regulates T_3-stimulated OC synthesis in osteoblasts.

Introduction

Bone metabolism is a highly coordinated process performed mainly by two types of functional cells, osteoblasts and osteoclasts. The former is responsible for bone formation and the latter for bone resorption. At present, it is well recognized that osteoblasts play a pivotal role in the regulation of bone resorption through receptor activator of nuclear factor-κB ligand (RANKL) expression which is responsive to numerous bone resorptive stimuli (1). Osteoblasts, which are differentiated from mesenchymal progenitors, express various cell type-specific markers during the differentiation process. Osteocalcin (OC) is a bone-specific protein that is modified post-translationally by vitamin K-dependent γ-carboxylation, known as bone Gla-protein (2). It is well known that OC is synthesized specifically in osteoblasts; therefore, OC is recognized as one of the markers of the mature osteoblast phenotype. In contrast, OC-deficient mice reportedly display an increase in bone formation without impairing bone resorption, suggesting that OC is a determinant of moderate bone formation (3). In addition, it has recently been shown that un-carboxylated OC released from osteoblasts regulates energy metabolism through acting on pancreatic β-cells to increase insulin synthesis, adipocytes to increase adiponectin and skeletal myocytes to glucose uptake (4,5). These findings lead us to speculate that bone, as an endocrine organ, plays a vital role also in energy metabolism through the release of OC. However, the exact mechanism underlying OC synthesis in osteoblasts remains to be clarified.

Thyroid hormone is an important regulator of skeletal function as well as whole body metabolism. Thyroid hormone excess, namely hyperthyroidism, is a major cause of secondary osteoporosis (6). In the state of hyperthyroidism, the serum levels of alkaline phosphatase and OC, markers of the osteoblast phenotype, are elevated as well as the excretion of pyridinoline and hydroxypyridinoline cross-link, which reflects bone resorption (6). It is known that an imbalance in bone remodeling causes the loss of bone mass by hyperthyroidism (6). The receptors for triiodothyronine (T_3), a member of the steroid hormone receptor superfamily, are expressed in osteoblasts (6). It has been reported that thyroid hormone stimulates alkaline phosphatase activity and secretion of OC and insulin-like growth factors in osteoblasts and that it modulates proliferation of osteoblasts (6-9). In our previous studies (10,11), we reported that p38 mitogen-activated protein (MAP) kinase but not p44/p42 MAP kinase, is involved in

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the T_3-stimulated OC synthesis in osteoblast-like MC3T3-E1 cells, and that the adenylyl cyclase-cAMP system has an inhibitory role in OC synthesis via suppression of p38 MAP kinase activation.

AMP-activated protein kinase (AMPK) is generally known to regulate multiple metabolic pathways (12). AMPK has emerged over the last decade as a key sensing mechanism in the regulation of cellular energy homeostasis (13-15). The enzyme is activated by a variety of physiological and pathological stresses which increase the intracellular AMP:ATP ratio, either by increasing ATP consumption or by decreasing ATP production in mammalian cells. Activated AMPK acts to restore cellular energy balance by ATP-generating pathways such as fatty acid oxidation, while simultaneously inhibiting ATP utilizing pathways. Regarding bone metabolism, metformin, which can activate AMPK (16), reportedly increases markers of osteoblast differentiation including OC mRNA expression, and enhances mineralization in osteoblast-like MC3T3-E1 cells (17). It has recently been reported that AMPK activation regulates bone formation and bone mass (18). These previous findings led us to speculate that AMPK influences bone metabolism through the functional modulation of osteoblasts. We previously demonstrated that AMPK plays a role in the synthesis of vascular endothelial growth factor or interleukin-6 in osteoblasts (19,20). However, the exact role of AMPK in bone metabolism, particularly in osteoblasts, has not yet been fully elucidated.

In the present study, we investigated the mechanism behind T_3-stimulated OC synthesis in osteoblast-like MC3T3-E1 cells and the involvement of AMPK in OC synthesis. We here demonstrated that AMPK positively regulates T_3-stimulated OC synthesis in these cells.

Materials and methods

Materials. T_3 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse OC enzyme-linked immunosorbent assay (ELISA) kit was purchased from Biomedical Technologies, Inc. (Stoughton, MA, USA). Compound C, a pyrrazolopyrimidine derivative widely used as a specific and reversible AMPK inhibitor (16,21,22), was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). Phospho-specific AMPKα (Thr-172) antibodies, phospho-specific AMPKα (Ser-485) antibodies, AMPKα antibodies, phospho-specific AMPKβ (Ser-108) antibodies, phospho-specific AMPKβ (Ser-182) antibodies, AMPKβ antibodies and phospho-specific acetyl-CoA carboxylase antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). GAPDH antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An ECL Western blotting detection system was purchased from GE Healthcare, Ltd. (Buckinghamshire, UK). Control short interfering RNA (siRNA; Silencer Negative Control no. 1 siRNA) and AMPK-siRNA were purchased from Qiagen (Hilden, Germany). siLentFect was purchased from Bio-Rad (Hercules, CA, USA). TRIzol reagent and Omniscript Reverse Transcriptase kit were purchased from Invitrogen (Carlsbad, CA, USA) and Qiagen, respectively. Fast-start DNA Master SYBR-Green I was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Other materials and chemicals

were obtained from commercial sources. Compound C was dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the assay for OC or detection of the protein level by western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (23) were maintained as previously described (24). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm ($5x10^4$) or 90-mm ($2x10^5$) diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

OC assay. The cultured cells were stimulated by T_3 in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. The conditioned medium was collected at the end of the incubation, and the OC concentration was measured by an OC ELISA kit. When indicated, the cells were pretreated with various doses of compound C for 60 min.

Western blot analysis. Western blot analysis was performed as previously described (25). The cultured cells were stimulated by T_3 or vehicle in α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with various doses of compound C for 60 min. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl; pH 6.8, 3% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (26) on 10% polyacrylamide gel. The protein was fractionated and transferred onto Immun-Blot PVDF membranes (Bio-Rad). Membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween-20 (TBS-T; 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h before incubation with the primary antibodies. The following antibodies were used: phospho-specific AMPKα (Thr-172) antibodies, phospho-specific AMPKα (Ser-485) antibodies, AMPKα antibodies, phospho-specific AMPKβ (Ser-108) antibodies, phospho-specific AMPKβ (Ser-182) antibodies, AMPKβ antibodies, phospho-specific acetyl-CoA carboxylase antibodies and GAPDH antibodies as primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG [Kirkegaard & Perry Laboratories (KPL), Inc., Gaithersburg, MD, USA] were used as secondary antibodies. The primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dry milk in TBS-T. Peroxidase activity on the membrane was visualized on X-ray film by means of the ECL Western blotting detection system.

Real-time RT-PCR. The cultured cells were pretreated with compound C for 60 min and stimulated by T_3 for the indicated periods. Total RNA was isolated and transcribed into cDNA using TRIzol reagent and Omniscript Reverse Transcriptase kit. Real-time RT-PCR was performed using a LightCycler system (Roche Diagnostics) in capillaries and Fast-Start DNA Master SYBR-Green I provided with the kit. Sense and
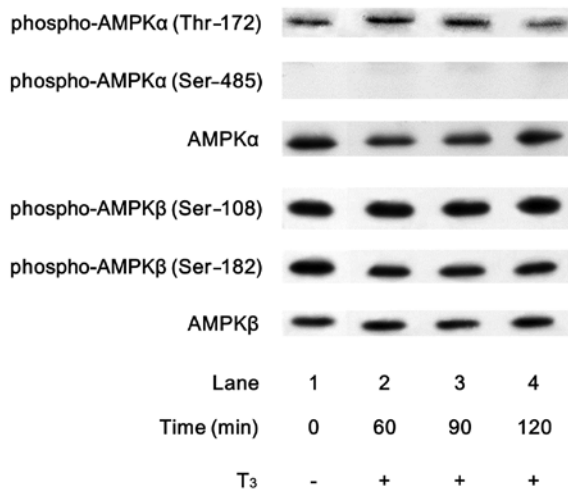
Figure 1. Effects of T_3 on the phosphorylation of AMPK in MC3T3-E1 cells. The cultured cells were stimulated by 10 nM T_3 for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific AMPKα (Thr-172), phospho-specific AMPKα (Ser-485), AMPKα, phospho-specific AMPKβ (Ser-108), phospho-specific AMPKβ (Ser-182) or AMPKβ.
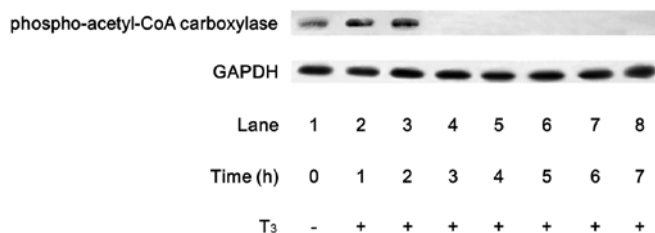

Figure 2. Effect of T_3 on the phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells. The cultured cells were stimulated by 10 nM T_3 for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific acetyl-CoA carboxylase or GAPDH.

antisense primers were synthesized based on the report of Zhang *et al* (27) for mouse OC mRNA and GAPDH mRNA. The amplified products were determined by melting curve analysis and agarose electrophoresis. OC mRNA levels were normalized with those of GAPDH mRNA.

siRNA transfection. To knockdown AMPK in MC3T3-E1 cells, the cells were transfected with negative control siRNA or AMPK-siRNA utilizing siLentFect according to the manufacturer's protocol. In brief, the cells ($1x10^5$ cells) were seeded into 35-mm diameter dishes in α-MEM containing 10% FCS and subcultured for 48 h. The cells were then incubated at 37°C with 50 nM siRNA-siLentFect complexes. After 24 h, the medium was replaced with α-MEM containing 0.3% FCS. The cells were then stimulated by T_3 in α-MEM containing 0.3% FCS for the indicated periods.

Determination of the enzyme activation. The absorbance of enzyme immunoassay samples was measured at 450 nm using the EL340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).
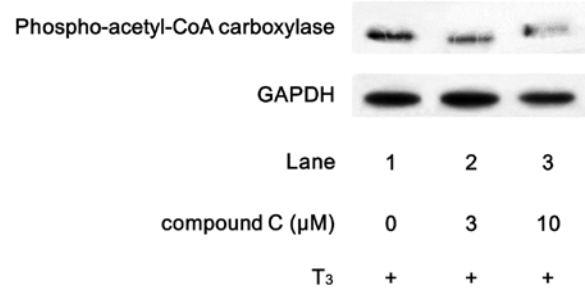

Figure 3. Effect of compound C on the T_3-induced phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of compound C for 60 min, and then stimulated by 10 nM T_3 for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific acetyl-CoA carboxylase or GAPDH.

Statistical analysis. The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $P<0.05$ was considered to indicate a statistically significant result. All data are presented as the means ± SEM of triplicate independent determinations.

Results

Effects of T_3 on the phosphorylation of AMPK in MC3T3-E1 cells. In order to investigate whether T_3 activates AMPK in osteoblasts, we first examined the effects of T_3 on the phosphorylation of AMPK in osteoblast-like MC3T3-E1 cells. T_3 markedly induced the phosphorylation of the AMPKα-subunit (Thr-172) (Fig. 1). The effect of T_3 on the phosphorylation of the AMPKα-subunit (Thr-172) reached its peak at 60-90 min and decreased thereafter. However, T_3 failed to affect the phosphorylation levels of AMPKα-subunit (Ser-485), AMPKβ-subunit (Ser-108) and AMPKβ-subunit (Ser-182) (Fig. 1).

Effect of compound C on the phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells. It is generally recognized that acetyl-CoA carboxylase, which catalyzes an important step in lipid synthesis, is a direct substrate of AMPK (15). We confirmed that T_3 markedly stimulated the phosphorylation of acetyl-CoA carboxylase in MC3T3-E1 cells (Fig. 2). The effect of T_3 on the acetyl-CoA carboxylase phosphorylation reached its peak within 2 h and decreased thereafter. We found that compound C, an inhibitor of AMPK (16), markedly suppressed the T_3-stimulated phosphorylation of acetyl-CoA carboxylase (Fig. 3).

Effect of compound C on the T_3-stimulated OC release in MC3T3-E1 cells. In order to clarify the involvement of AMPK in the T_3-stimulated OC release in osteoblasts, we next examined the effect of compound C on the release of OC stimulated by T_3 in MC3T3-E1 cells. Compound C, which alone did not affect the OC levels, significantly suppressed the T_3-stimulated OC release in a time-dependent manner (Fig. 4). The inhibitory effect of compound C was dose-dependent in the range between 0.3 and 10 μM (Fig. 5). The maximum effect of compound C was observed at 10 μM, which resulted in ~90% inhibition compared to the OC level with T_3 alone.
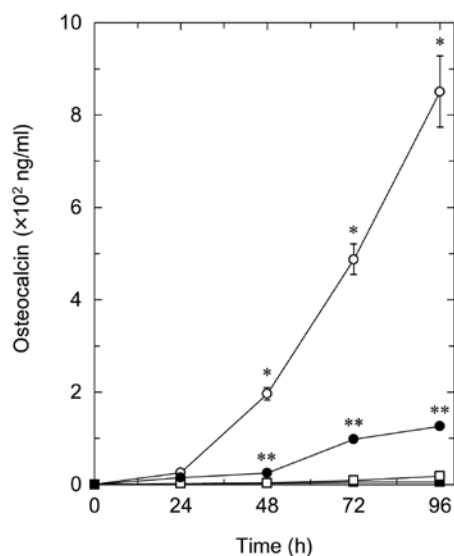
Figure 4. Effect of compound C on T_3-stimulated OC release in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM of compound C (closed symbols) or vehicle (open symbols) for 60 min, and then stimulated by 10 nM T_3 (circles) or vehicle (squares) for the indicated periods. Each value represents the mean ± SEM of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$P<0.05$, compared to the value of control; **$P<0.05$, compared to the value of T_3 alone.

Figure 6. Effect of compound C on T_3-induced OC mRNA expression in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM compound C or vehicle for 60 min, and then stimulated by 10 nM T_3 or vehicle for 48 h. Total RNA was isolated and transcribed into cDNA. The expression of OC mRNA and GAPDH mRNA was quantified by real-time RT-PCR. OC mRNA levels were normalized with those of GAPDH mRNA. Each value represents the mean ± SEM of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$P<0.05$, compared to the value of control; **$P<0.05$, compared to the value of T_3 alone.
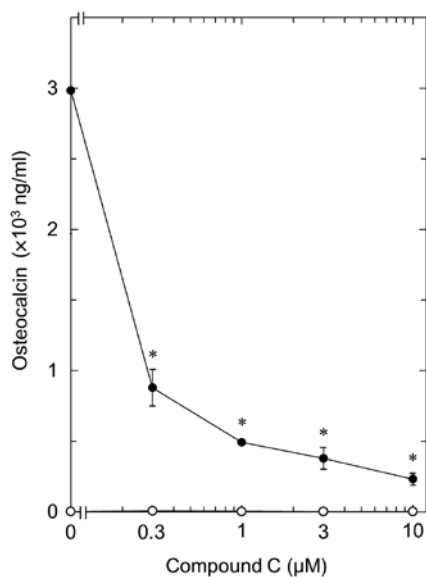

Figure 5. Dose-dependent effect of compound C on T_3-stimulated OC release in MC3T3-E1 cells. The cultured cells were pretreated with various doses of compound C for 60 min, and then stimulated by 10 nM T_3 (●) or vehicle (○) for 96 h. Each value represents the mean ± SEM of triplicate independent determinations. Similar results were obtained with two additional and different cell preparations. *$P<0.05$, compared to the value of T_3 alone.

Figure 7. Effect of AMPK siRNA on the T_3-stimulated OC release in MC3T3-E1 cells. The cultured cells were transfected with 50 nM control siRNA (cont) or 50 nM AMPK-siRNA (siRNA Mm_Prkaa1_1 Qiagen) by using the siLentFect. Forty-eight hours after transfection, the cells were stimulated by 10 nM T_3 or vehicle for another 96 h. OC concentrations of the culture medium were determined by ELISA. The osteocalcin level was corrected for the total protein level. Each value represents the mean ± SEM of triplicate independent determinations. *$P<0.05$, compared to the value of vehicle with control siRNA transfection; **$P<0.05$, compared to the value of T_3 with control siRNA transfection.

Effect of compound C on the T_3-induced OC mRNA expression in MC3T3-E1 cells. It has been shown that T_3 induces the mRNA expression of OC in osteoblasts (9). In order to investigate whether the suppression of T_3-stimulated OC synthesis by compound C is mediated through transcriptional events, we next examined the effect of compound C on T_3-induced OC mRNA expression. Compound C, which by itself had little
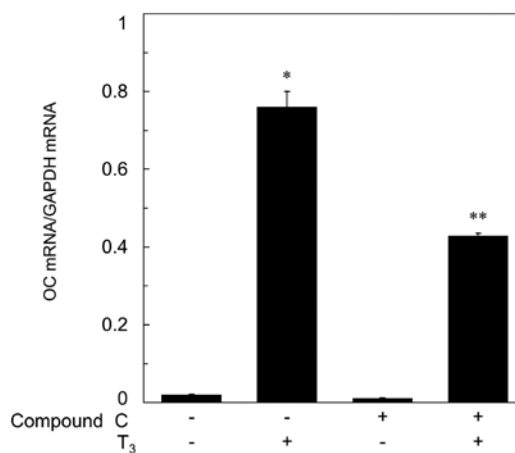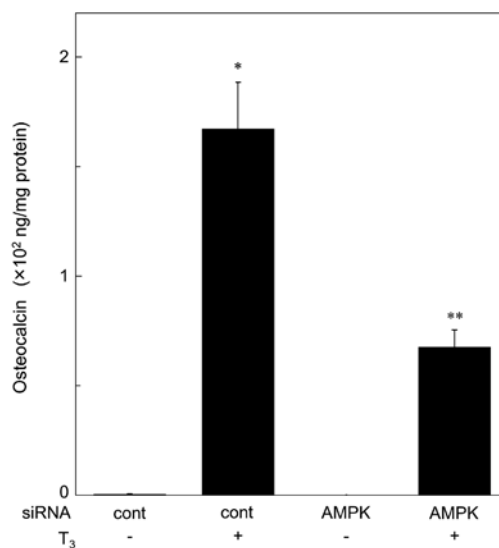
effect on the basal level of OC mRNA, significantly reduced the T_3-induced level of OC mRNA (Fig. 6).

Effect of AMPK-siRNA on T_3-stimulated OC release in MC3T3-E1 cells. We further investigated the effect of AMPK knockdown on the OC release stimulated by T_3 in MC3T3-E1 cells. In comparison with the control cells, the T_3-stimulated OC release was significantly diminished in AMPK-knockdown

cells by AMPK-siRNA (Fig. 7). Approximately 60% suppression of the T_3-stimulated OC release resulted following the treatment with AMPK-siRNA.

Discussion

In the present study, we showed that T_3 induced the phosphorylation of AMPK in osteoblast-like MC3T3-E1 cells, using phospho-specific AMPKα-subunit (Thr-172) antibodies. It is generally recognized that the phosphorylation of Thr-172 in the AMPKα-subunit is necessary for AMPK activity (12,28). Therefore, it is likely that T_3 stimulates the activation of AMPK in osteoblast-like MC3T3-E1 cells. To the best of our knowledge, this is probably the first report showing the involvement of AMPK in the intracellular signaling of T_3 in osteoblasts. On the other hand, T_3 failed to induce the phosphorylation of AMPKα-subunit (Ser-485). It seems unlikely that the phosphorylation of Ser-485 in the α-subunit is involved in T_3-induced AMPK activation in MC3T3-E1 cells. In addition, we found that AMPKβ-subunits (Ser-108 and Ser-182), which were phosphorylated without stimulation, were barely affected by T_3. It has been shown that phosphorylation at Ser-108 of the AMPKβ-subunit is required for the activation of AMPK, while phosphorylation of Ser-182 affects AMPK localization (29). Moreover, we showed here that the phosphorylation of acetyl-CoA carboxylase, known as a direct substrate of AMPK (30), was significantly induced by T_3. The time course of the phosphorylation of AMPKα-subunit (Thr-172) stimulated by T_3 appears to be more rapid than that of acetyl-CoA carboxylase. Furthermore, we found that compound C, an AMPK inhibitor (16), reduced the phosphorylation levels of acetyl-CoA carboxylase by T_3. It is probable that the inhibitory effect of compound C is exerted through the suppression of AMPK. Based on our findings, it is most likely that T_3 positively regulates AMPK activity via the phosphorylation of the AMPKα-subunit (Thr-172) in osteoblast-like MC3T3-E1 cells.

We previously demonstrated that T_3 induces OC synthesis which is a marker of osteoblast differentiation in osteoblast-like MC3T3-E1 cells (10,11). Thus, we next investigated whether or not AMPK is involved in the T_3-stimulated OC synthesis in MC3T3-E1 cells. We showed that the T_3-stimulated OC release was significantly suppressed by compound C, an inhibitor of AMPK (16), suggesting that AMPK is involved in the OC release induced by T_3 in these cells. Additionally, compound C markedly reduced the OC mRNA expression induced by T_3, suggesting that AMPK regulates OC synthesis at a point upstream of the transcriptional process. We further examined the effect of AMPK knockdown by siRNA on T_3-stimulated OC release and demonstrated the concomitant reduction in T_3-stimulated induction of OC by AMPK knockdown in the osteoblast-like MC3T3-E1 cells. Taking our findings into account, it is most likely that AMPK functions as a positive regulator in T_3-stimulated OC synthesis in osteoblasts.

Thyroid hormone excess, or hyperthyroidism, is a major cause of secondary osteoporosis. Both bone formation and bone resorption are accelerated in the state of hyperthyroidism, and the predominance of bone resorption rather than bone formation results in the loss of bone mass (6). On the other hand, deficiency of the thyroid hormone causes severe skeletal growth retardation in infants. These clinical states indicate the importance of the thyroid hormone in bone metabolism. Thus, it is essential to clarify the precise mechanism of T_3 action, an active form of thyroid hormone, particularly in osteoblasts which promote bone formation and regulate bone resorption. OC, a γ-carboxylated calcium-binding protein, is generally recognized to be produced by mature osteoblasts (2). It is well known that the serum level of OC is elevated in patients with hyperthyroidism (6). OC-deficient mice reportedly develop hyperostosis (3), suggesting its crucial role in the bone remodeling process. Thus, our present findings demonstrating the AMPK-dependent OC release induced by T_3 provide a new concept of bone metabolism. In addition, it has recently been shown that un-carboxylated OC released from osteoblasts as a bone-derived hormone regulates energy metabolism by acting on pancreatic β-cells to increase insulin synthesis, on adipocytes to increase adiponectin and on skeletal myocytes for glucose uptake in rodent (4,5). This significant effect of un-carboxylated OC has not yet been clarified in human; however, osteoblast-releasing OC may participate in whole body energy regulation. In addition, metformin, an AMPK-activating substance, has already been widely used as an agent for diabetes. AMPK plays a vital role as an energy sensor promoting the development of tissue derived from mesenchymal cells such as muscle and bone. Taking our present findings into account as a whole, AMPK regulation of OC release by osteoblasts may be a therapeutic target for metabolic disorders. Further investigation is required to clarify the details of AMPK in bone metabolism. In conclusion, our results strongly suggest that AMPK positively regulates T_3-stimulated OC synthesis in osteoblasts.

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