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Overview of Calpain-mediated Regulation of Bone and Fat mass in Osteoblasts

Masako Shimada, MD, Ph.D

Endocrine Unit, Massachusetts General Hospital and Department of Medicine, Harvard Medical School, Boston, Massachusetts, 02114

Abstract

The receptor for parathyroid hormone (PTH) and PTH-related peptide (PTH1R) belongs to the class II G protein-coupled receptor (GPCR) superfamily. The calpain small subunit encoded by the gene *Capns1* is the second protein and the first enzyme identified by a yeast two-hybrid screen using the intracellular C-terminal tail of the rat PTH1R. The calpain regulatory small subunit forms a heterodimer with the calpain large catalytic subunit and modulates various cellular functions as a cysteine protease. To investigate a physiological role of the calpain small subunit in cells of the osteoblast lineage, we generated osteoblast-specific *Capns1* knockout mouse models and characterized their bone phenotype. Molecular mechanisms by which calpain modulates cell proliferation of the osteoblast lineage were further examined *in vitro*. Moreover, we utilized the mutant mice as a disease model of osteoporosis accompanied with impaired bone resorptive function and suggested a possible clinical translation of our basic research finding.

Keywords

calpain; osteoblasts; fat; glucose metabolism; cell cycle

Introduction

Several lines of recent evidence have established a new paradigm, a cross talk among bone, fat, and glucose homeostasis *in vivo*. Karsenty's group first showed that an adipokine, leptin, decreases bone mass through a mechanism mediated by the sympathetic nervous system [1–3] and serotonin-mediated signaling [4] in the brain. Uncarboxylated osteocalcin, an active form of bone-derived hormone, osteocalcin, was next shown to regulate body adiposity and glucose homeostasis through its regulation of adiponectin levels [5–7].

The calpain small subunit encoded by the gene *Capns1* is the second protein to the sodium hydrogen exchanger regulatory factors [8] identified as a molecule other than G-proteins which binds directly to the PTH1R (class II GPCR) [9] and markedly alters its receptor function [10]. The ubiquitously expressed Calpains 1 and 2 belong to a family of calcium-dependent intracellular cysteine proteases [11, 12]. Both calpains are heterodimers consisting of a large catalytic subunit and a small regulatory subunit. Ablation of the calpain small subunit eliminates activities of calpains [13] and leads to embryonic lethality in mice [13, 14]; these results suggest essential roles of the calpain small subunit for calpain activity and stability and during embryonic development. *In vitro* evidence previously suggested that calpain plays an important role in PTH-mediated cellular functions in osteoblasts. PTH

increases calpain activities in osteoblasts [15, 16] and pretreatment of MC3T3-E1 osteoblastic cells with chemical calpain inhibitors blocks PTH-stimulated cell proliferation and differentiation [17, 18]. However, an *in vivo* role of the calpain small subunit in cells of the osteoblast lineage was poorly understood. Here I summarize the recent progress of our study on a role of calpain in cells of the osteoblast lineage in regulation of bone and fat mass and glucose homeostasis *in vivo*.

1. The calpain small subunit directly binds to the intracellular C-terminal tail of rat (r) PTH1R and modulates PTH-mediated receptor signaling

PTH1R binds the calpain small subunit through an N-terminal portion of the intracellular C-tail that requires, but not limited to, amino acids W474, S475, and W477 of the rPTH1R. This interaction site, the W-R[W] sequence of the rPTH1R, is highly conserved among PTH1Rs in different species and among other type II GPCRs including receptors for pituitary adenylate cyclase-activating polypeptide, secretin, and calcitonin.

Calpains are cysteine proteases that modulate various cellular functions by cleaving substrate proteins [11]. We showed calpain, at least partially, hydrolyzes PTH1R in calcium- and PTH-dependent manners. Moreover, reduction of calpain activity decreased PTH-stimulated cAMP accumulation in embryonic fibroblasts and MC3T3-E1 osteoblastic cells [10], and PTH-stimulated mRNA expression of *c-fos*, a downstream target gene of PTH [19]. Collectively, these results established a direct binding of the calpain small subunit to the rPTH1R, and suggested that calpain modulates calcium-dependent hydrolysis of the PTH1R and PTH-mediated receptor signaling.

2. Osteoblast-specific *Capns1* knockout mice developed an osteoporotic bone phenotype

To examine a physiological role of calpain in cells of the osteoblast lineage, we generated two conditional *Capns1* knockout mice in cells of the osteoblast lineage using the Cre-loxP system. Osterix is a transcription factor of the specificity protein gene family and a putative master regulator for osteoblast differentiation [20]. Osterix functions upstream of collagen Ia1 in cells of the osteoblast lineage (Table 1). Therefore, we could disrupt *Capns1* expression in osteoblasts and pre-osteoblasts by using mice expressing Cre-recombinase under the control of 2.3 kb-*collagen Ia1* [21] and *osterix* promoters [22], respectively. We found no significant differences in gross skeletal phenotype including body weight, size, and bone histology between mice with *collagen Ia1* promoter-driven ablation of *Capns1* and their control littermates (Fig. 1A and 1C). However, mutant mice with targeted deletion of *Capns1* under the control of *osterix* promoter exhibited lower body weight than control mice and a significant reduction of both trabecular and cortical bone (Figs. 1B and 1D). This osteoporotic bone phenotype of the mutant mice was associated with a severe impairment of osteoblast proliferation and differentiation rather than increased osteoblast apoptosis and increased osteoclast number and activity [19]. Reduced serum levels of total osteocalcin (a marker for bone formation) and tartrate-resistant acid phosphatase 5b (a bone resorption marker) of the mutant mice also supported their bone phenotype [10]. Collectively, our finding is the first *in vivo* demonstration that the calpain small subunit plays a critical role in cells of the osteoblast lineage earlier than osteoblasts. Thereafter, mutant mice with targeted deletion of *Capns1* under the control of *osterix* promoter are referred to osteoblast-specific *Capns1* knockout mice.

3. Calpain in osteoblasts regulates cell proliferation through its regulation of cyclin-dependent kinase inhibitor 1b (p27^{Kip1})

To examine an underlying mechanism, by which calpain regulates cell proliferation in osteoblast-specific *Capns1* knockout mice, we created osteoblastic cell lines stably expressing *Capns1* RNA interference (*Capns1* knockdown cells) [23]. Figure 2 summarizes our *in vitro* findings. Major target molecules of calpain in cells of the osteoblast lineage are the regulatory subunits of protein phosphatase 2A (PP2A) and p27^{Kip1}. (i) Bertoli et al previously showed that ablation of *Capns1* reduces calpain activity and thereby stabilizes PP2A. Increased binding of PP2A to Akt reduces phosphorylation of Akt and then that of forkhead Box O (FoxO) 3A protein. Unphosphorylated FoxO3A protein was shown to translocate to nucleus to increase p27^{Kip1} mRNA and p27^{Kip1} protein levels [24]. Our data also demonstrated an increased binding of PP2A and Akt, thereby reduced phosphorylation of Akt and FoxO3A, and increased p27^{Kip1} mRNA levels in *Capns1*-knockdown osteoblasts [23]. Moreover, it has been reported that calpain-mediated degradation of p27^{Kip1} plays an important role in allowing a cyclin D-cdk4 complex to phosphorylate the retinoblastoma tumor suppressor, Rb, on threonine 821 and disrupt its binding to transcription factor, E2F; this, in turn, promotes cell cycle progression from G1 to S phase [25, 26]. Phosphorylation of p27^{Kip1} on Serine10 has been suggested to signal a nucleocytoplasmic translocation of p27^{Kip1} [27, 28], which is critical for mitogen-activated protein kinase-dependent p27^{Kip1} degradation by calpain [29]. Consistent with those and our reports [30], *Capns1*-knockdown cells of the osteoblast lineage revealed (ii) accumulated cell cycle proteins known as calpain substrates, including cyclin D and p27^{Kip1}; (iii) time-dependent accumulation of phosphorylated p27^{Kip1} protein on Serine 10; (iv) reduced phosphorylation of Rb on threonine 821; and (v) impaired cell cycle transition from G1 to S phase [23]. Collectively, these findings suggest that ablation of calpain in cells of the osteoblast lineage modulates both transcription and degradation of p27^{Kip1}, resulting in an increase of p27^{Kip1} protein levels and cell cycle arrest at the G1/S transition.

4. Osteoblast-specific *Capns1* knockout mice developed a high-fat diet-induced excess body weight gain and impaired glucose tolerance

As described above, osteoblast-specific *Capns1* knockout mice exhibited severe osteoporotic bone phenotype mainly due to reduced cell proliferation of osteoblast lineage. Associated with the decreased osteoblast number, mutant bones also showed reduced osteoclast number and activity, and their limited ability for bone resorption [19]. Moreover, reduced serum levels of total osteocalcin in mutant mice were mainly due to reduced levels of an active form of osteocalcin, uncarboxylated osteocalcin [31]. Uncarboxylated osteocalcin was shown to positively regulate adiponectin levels and glucose homeostasis [5]. Serum uncarboxylated osteocalcin levels increase dependently on osteoclastic bone resorption subsequent to insulin-stimulated receptor signaling in osteoblasts [7]. Therefore, we interpreted that reduced levels of uncarboxylated osteocalcin of mutant mice are presumably due to their reduced bone resorptive function. Indeed, osteoblast-specific *Capns1* knockout mutant mice are resistant to a low calcium diet-stimulated bone resorption [31].

To examine how the bone phenotype of mutant mice and their reduced serum uncarboxylated osteocalcin levels affect a balance between bone and fat mass, we provided mice with a high fat diet. Despite their lean phenotype on a normal chow diet, mutant mice gained more weight than control littermates and showed significantly increased serum levels of leptin, an indicator of body adiposity. Mutant mice also showed reduced adiponectin and increased resistin levels, known regulators of glucose metabolism in mice, and impaired glucose tolerance in glucose tolerance test [23]. Taken together, these results suggested that

mice with osteoporosis and reduced bone resorptive function are susceptible to increase body adiposity and develop abnormal glucose homeostasis under a high fat diet presumably through a mechanism mediated by reduced uncarboxylated osteocalcin levels (Fig. 3). Our finding could apply to patients with osteoporosis taking medication, which inhibits bone resorption; dietary management could play a critical role for prevention of metabolic diseases among the patients with osteoporosis [23].

5. Conclusion and Future directions

Our investigation began with a finding that the calpain small subunit interacts with PTH1R in a yeast two-hybrid screen. A detailed analysis using tissue-specific knockout mouse models revealed a physiological role of *Capns1* in cells of the osteoblast lineage, which led us to further uncover a molecular mechanism by which the calpain subunit regulates cell proliferation of the osteoblast lineage. Moreover, we used the mutant mice as a disease model of osteoporosis accompanied with limited ability for bone resorption and made an observation, which could lead to a prevention of metabolic diseases in patients suffering from osteoporosis in the future clinical practice.

Despite the significant progress we have made, there remain many questions to be answered. We have not yet fully understood, for example, how the increased p27^{Kip1} protein levels by calpain inhibition in cells of the osteoblast lineage affects an efficiency of anabolic action of PTH in treatment of osteoporosis. A line of evidence has suggested that upregulation of p27^{Kip1} and p21^{Cip1} proteins [32, 33] through PTH-mediated increase of protein kinase A (PKA) signaling is positively associated with an anabolic action of PTH in bone [32]. Because we have shown that calpain uniquely increases p27^{Kip1} protein levels, at least partially, through a mechanism independent of a PTH–PKA signaling pathway [23], bone with reduced calpain activity may increase an efficiency of anabolic action of PTH. Moreover, loss of p27^{Kip1} is observed in some types of carcinoma cells including metastatic canine mammary carcinomas [34, 35]. Therefore, calpain inhibition-mediated increase of p27^{Kip1} protein might delay initiation and progress of those primary cancers and their metastasis.

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Figure 1. Calpain plays more critical roles in cells of the osteoblast lineage earlier than osteoblasts in mice

No significant phenotypic differences in bone were found between Collagen Iα1- *Capns1*-KO and Collagen Iα1-*Control* mice; while, *Osx*- *Capns1*-KO mutant mice showed markedly reduced trabecular bone compared with *Osx*-*Control* mice. H&E stained tibial bones isolated from Collagen Iα1-*Capns1*-KO and Collagen Iα1-*Control* mice. Brackets show trabecular bone areas. B. *In situ* hybridization analysis using *Collagen Iα1* probe on sections of 2-week-old proximal tibias of *Osx*- *Capns1*-KO and *Osx*- *Control* mice. *Osx*- *Capns1*-KO mutant mice showed smaller bone and reduced trabecular bone than controls. C. No differences in body weight was observed between 1- and 2-week old Collagen Iα1- *Capns1*-KO and Collagen Iα1-*Control* mice. D. *Osx*- *Capns1*-KO mice showed significantly reduced body weight at birth and 2 weeks of age compared with *Osx*- *Control* littermates. *Osx*, osterix. KO, knockout.

Figure 2. Calpain-mediated regulation of p27^{Kip1} protein and cell cycle progression in cells of the osteoblast lineage

(i) Calpain inhibition stabilizes PP2A, increases binding of PP2A to Akt, and reduces phosphorylation of Akt and then FoxO3A proteins. Unphosphorylated FoxO3A protein was shown to translocate to nucleus to increase *p27^{Kip1}* mRNA and p27^{Kip1} protein levels. *Capns1* knockdown osteoblasts (ii) accumulate cell cycle proteins known as calpain substrates including cyclin D and p27^{Kip1}, (iii) impair phosphorylation of p27^{Kip1} on serine 10 and reduce degradation of p27^{Kip1} protein. Increased p27^{Kip1} protein inactivates cyclin D-cdk 4 complex, thereby (iv) reduces phosphorylation of Rb, which leads to impaired transition from G1 to S phase of the cell cycle. Calpain substrates are underlined.

Figure 3. A mechanism, by which osteoblast specific *Capns1* knockout mice on a high fat diet developed glucose intolerance and a possible clinical implication of the finding

A characteristic bone phenotype of osteoblast-specific *Capns1* knockout mice was severe osteoporosis with reduced bone resorptive function. This phenotype was similar to that of patients with osteoporosis receiving anti-resorptive agents. Mutant mice and those patients show reduced uncarboxylated osteocalcin levels. A high fat diet promoted excess gain of body weight, increased serum levels of leptin and resistin, and reduced adiponectin levels in mutant mice. Moreover, consistent with their increased resistin and reduced adiponectin levels, mutant mice developed impaired glucose tolerance. We suggest that dietary management could be critical for patients with osteoporosis receiving medication, which reduces bone resorption, even if they show normal body weight and glucose homeostasis before the treatment.

Table 1

Cells of the osteoblast lineage and Gene markers

Osteoblast lineage	Mesenchymal	Osteoprogenitor	Pre-osteoblast	Osteoblast	Mature osteoblast
Gene marker	Prx-1	Runx2	Osterix	Collagen Ia1	Osteocalcin

Prx-1; paired-related homeobox gene 1, Runx: Runt-related transcription factor 2