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Inhibition of CaMKK2 reverses age-associated decline in bone mass

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ABSTRACT

Decline in bone formation is a major contributing factor to the loss of bone mass associated with aging. We previously showed that the genetic ablation of the tissue-restricted and multifunctional Ca^{2+}/calmodulin (CaM)-dependent protein kinase kinase 2 (CaMKK2) stimulates trabecular bone mass accrual, mainly by promoting anabolic pathways and inhibiting catabolic pathways of bone remodeling. In this study, we investigated whether inhibition of this kinase using its selective cell-permeable inhibitor STO-609 will stimulate bone formation in 32 week old male WT mice and reverse age-associated of decline in bone volume and strength. Tri-weekly intraperitoneal injections of saline or STO-609 (10 μM) were performed for six weeks followed by metabolic labeling with calcein and alizarin red. New bone formation was assessed by dynamic histomorphometry whereas micro-computed tomography was employed to measure trabecular bone volume, microarchitecture and femoral mid-shaft geometry. Cortical and trabecular bone biomechanical properties were assessed using three-point bending and punch compression methods respectively. Our results reveal that as they progress from 12 to 32 weeks of age, WT mice sustain a significant decline in trabecular bone volume, microarchitecture and strength as well as cortical bone strength. However, treatment of the 32 week old WT mice with STO-609 stimulated apposition of new bone and completely reversed the age-associated decrease in bone volume, quality, as well as trabecular and cortical bone strength. We also observed that regardless of age, male *Camkk2*$^{-/-}$ mice possessed significantly elevated trabecular bone volume, microarchitecture and compressive strength as well as cortical bone strength compared to age-matched WT mice, implying that the chronic loss of this kinase attenuates age-associated decline in bone mass. Further, whereas STO-609 treatment and/or the absence of CaMKK2 significantly enhanced the femoral mid-shaft geometry, the mid-shaft cortical wall thickness and material bending stress remained similar among the cohorts, implying that regardless of treatment, the material properties of the bone remain similar. Thus, our cumulative results provide evidence for the pharmacological inhibition of CaMKK2 as a bone anabolic strategy in combating age-associated osteoporosis.

Introduction

The ubiquitous second messenger Ca^{2+} connects extracellular signals to intracellular biological processes [1,2]. Ligand–receptor interactions at the cell surface generate intracellular Ca^{2+} transients that are immediately recognized by the highly conserved calmodulin (CaM). The resulting Ca^{2+}/CaM complex binds to and activates a host of downstream targets including the multifunctional Ca^{2+}/CaM-dependent protein kinases (CaMKs) [3]. CaMKs are a family of serine/threonine protein kinases that includes CaMKI, CaMKII and CaMKIV [2,4,5]. Of these, CaMKII is fully activated through autophosphorylation following Ca^{2+}/CaM binding. The other two family members CaMKI and CaMKIV require phosphorylation of an activation-loop threonine by two upstream kinases CaMKK1 (α) and CaMKK2 (β). The resulting CaMK signaling cascade has important functions in cell cycle, cell differentiation, adaptive and innate responses by immune cells, thymocyte survival, hematopoietic stem cell homeostasis, cerebellar granular cell differentiation, axon guidance, learning and memory [4–15]. More recently, adenosine mono-phosphate activated protein kinase (AMPK), a protein that coordinates cellular energy balance was

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identified as the third substrate of CaMKK2 [1,16]. CaMKK2 is tissue-restricted and its loss protects mice from diet-induced obesity, insulin resistance and inflammatory responses [16–18].

We recently reported that CaMKK2 is expressed in osteoblasts (OBs) and osteoclasts (OCs) and that mice lacking this kinase possess enhanced trabecular bone mass in their long bones, along with significantly elevated numbers of OBs and fewer multinuclear OCs [19]. Moreover, pharmacological inhibition of CaMKK2 activity using its selective, cell-permeable pharmacological inhibitor STO-609 stimulated OB differentiation while inhibiting OC differentiation in vitro [19,20]. Furthermore, STO-609 treatment protected 10 week-old mice from ovariectomy-induced bone loss by stimulating OBs and inhibiting OCs in vivo [19]. Collectively, our initial studies demonstrated that the loss of CaMKK2 strongly favors the anabolic pathways of remodeling and that its pharmacological inhibition protects mice from post-menopausal osteoporosis.

In the present study, we wanted to extend these observations by evaluating whether pharmacological inhibition of CaMKK2 will reverse age-related decline in bone mass, which occurs mainly due to decreased bone formation by OBs, rather than an increase in bone resorption [21]. Similar to humans, the trabecular bone volume in male C57BL6 mice declines continuously from 1.5 months to 24 months of age and the most rapid decline is observed between 6 and 12 months of age [22,23]. Accordingly, we surmised that the acute inhibition of CaMKK2 using STO-609 would trigger new bone formation and reverse the age-associated loss of trabecular bone volume suffered by male C57BL6 mice at 32 weeks or 8 months of age. We also investigated whether the age-associated decline in trabecular bone microarchitecture as well as weakening of trabecular and cortical bone biomechanical strength are rescued following STO-609 treatment of 32 week old mice.

Materials and methods

Mice

Male WT and *Camkk2*$^{-/-}$ mice (C57BL6 background) were housed in the University of Louisville (UofL) Baxter II Vivarium under a 12-h light, 12-h dark cycle. Food and water were provided ad libitum. All care and experimental procedures were performed according to UofL Institutional Animal Care and Use Committee protocols and in compliance with NIH guidelines on the use and care of laboratory and experimental animals.

Reagents and treatments

STO-609 was purchased from TOCRIS Bioscience (Ellsville, MO, USA) and prepared as mentioned previously [19]. Tri-weekly intraperitoneal (i.p.) injections of 200 μl per mouse of either saline (n = 9) or 10 μM STO-609 (n = 9) were administered into 32 week old WT mice for 6 weeks. Seven and two days before euthanasia, the saline (n = 5) and STO-609 treated mice (n = 8) were injected via i.p. with calcein (5 mg/ml) and alizarin red (15 mg/ml) respectively, at 100 μl per mouse. Long bones were harvested; tibiae were utilized for dynamic histomorphometry and femurs were utilized for microcomputed tomography (μCT) and mechanical testing. Microarchitecture analyses as well as assessment of both cortical and trabecular bone strength were performed. Untreated 12 week old WT (n = 11) and *Camkk2*$^{-/-}$ (n = 11) mice as well as 32 week old *Camkk2*$^{-/-}$ mice (n = 6) were used as controls for μCT and strength analyses.

Dynamic bone histomorphometry and polarizing microscopy

Undecalcified histology and dynamic histomorphometry were performed on tibiae that were fixed in 70% ethanol. Longitudinal sections of the tibiae (10 μm) were prepared and new bone formation was assessed by fluorescence microscopy of calcein (green) and alizarin red (red). The region of interest was the metaphyseal bone covering 3.0 mm beneath the growth plate [24]. Histomorphometric analysis was performed by a blinded observer using BioQuant OSTEO 2010 software (BioQuant Image Analysis Corporation) and standard parameters [25]. The sections were subsequently stained with picrosirius red and imaged using polarizing microscopy to visualize spatial distribution of collagen fibers in bone [26].

Micro-CT imaging

Micro-computed tomography (μCT) imaging was performed on a high resolution CT scanner (Actis HR225-150; BIR, Lincolnshire, IL, USA) located at the University of Louisville Orthopaedic Bioengineering Lab. Up to 3 femurs were imaged simultaneously at an isotropic voxel size of 7 μm. Transverse images of femurs covering a length of approximately 5.25 mm were processed individually for each femur using a combination of two-dimensional (ImageJ, NIH Image, Bethesda, MD) and three-dimensional (VG Studio Max, Volume Graphics, Heidelberg, DE) imaging software. We cropped a box-shaped volume of interest (VOI) approximately 2.0 × 1.0 × 0.75 mm from this stack of images. The VOI was located within the distal femoral metaphysis extending to within 100 μm of the proximal side of the distal femoral physis. After using a 2 × 2 Gaussian filter and a histogram based single gray-level threshold to segment cortical and cancellous bone tissue from background and soft tissue, trabecular architectural properties including bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were determined for a standardized region in the distal femoral metaphysis. Mid-shaft geometry was calculated by measuring the mediolateral and anterior–posterior inner and outer diameters of the mid-diaphysis from the μCT images and calculating the cross-sectional area MOI and cortical bone thickness from these measurements using an elliptical cross-section assumption.

Mechanical testing

After the femurs were imaged in the μCT scanner, they were prepared for testing to determine cortical bone mechanical properties using three-point bending and cancellous bone mechanical properties using a flat-tipped cylindrical punch. The femurs were kept moist by wrapping in saline soaked gauze from the time of collection up to the time of testing. A servohydraulic load frame equipped with a 500 N capacity load cell with a sensitivity of 0.2 N was used for both mechanical tests (Model 858 Bionix, MTS Corp., Eden Prairie, MN).

Three-point-bending was performed by placing the femur across an 8 mm wide support span and loading vertically with a rounded knife edge at the center of the span contacting the posterior surface of the femoral diaphysis. The loading was applied at a displacement rate of 1 mm/min until the femur failed. The amount of force, F (N), applied was recorded and the magnitude of the bending moment at the point of failure was calculated as:

$$M = \left[\frac{1}{4} * F * \text{support width}\right] (N * mm).$$

Following the three-point bending test, the distal portion of the same femur was prepared for a punch-type compression test to measure the strength of the cancellous bone material in the distal part of the femoral metaphysis. To perform this test in mice, we modified a procedure outlined by An et al. for flat head indentation of the distal femur cancellous bone in rats [27]. The thickness of the epiphysis and distance to the base of the epiphyseal plate was measured for individual bones using μCT image analysis. This information was employed to trim the distal femurs to expose the base of the epiphyseal plates for precise indentation (Fig. 3Ci). Briefly, the distal femur was mounted in the head of