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Methionine restriction alters bone morphology and affects osteoblast differentiation

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ABSTRACT

Methionine restriction (MR) extends the lifespan of a wide variety of species, including rodents, drosophila, nematodes, and yeasts. MR has also been demonstrated to affect the overall growth of mice and rats. The objective of this study was to evaluate the effect of MR on bone structure in young and aged male and female C57BL/6J mice. This study indicated that MR affected the growth rates of males and young females, but not aged females. MR reduced volumetric bone mass density (vBMD) and bone mineral content (BMC), while bone microarchitecture parameters were decreased in males and young females, but not in aged females compared to control-fed (CF) mice. However, when adjusted for bodyweight, the effect of MR in reducing vBMD, BMC and microarchitecture measurements was either attenuated or reversed suggesting that the smaller bones in MR mice is appropriate for its body size. In addition, CF and MR mice had similar intrinsic strength properties as measured by nanoindentation. Plasma biomarkers suggested that the low bone mass in MR mice could be due to increased collagen degradation, which may be influenced by leptin, IGF-1, adiponectin and FGF21 hormone levels. Mouse preosteoblast cell line cultured under low sulfur amino acid growth media attenuated gene expression levels of *Col1al*, *Runx2*, *Bglap*, *Alpl* and *Spp1* suggesting delayed collagen formation and bone differentiation. Collectively, our studies revealed that MR altered bone morphology which could be mediated by delays in osteoblast differentiation.

1. Introduction

Methionine restriction (MR) extends the lifespan of a wide variety of species, including rodents, drosophila, nematodes, and yeasts (Brown-Borg et al., 2014; Cabreiro et al., 2013; Johnson and Johnson, 2014; Lee et al., 2014; Miller et al., 2005; Orentreich et al., 1993; Richie et al., 1994; Ruckenstuhl et al., 2014). Lifespan extension by MR in rodents could be due to several factors, including: 1) delays in age-related diseases, such as obesity and diabetes (Ables et al., 2012; Perrone et al., 2008; Plaisance et al., 2011; Stone et al., 2014); 2) decreased mitochondrial oxidative stress (Caro et al., 2008, 2009; Sanchez-Roman and Barja, 2013); and 3) reduced risk for cancer progression (Komninou et al., 2006; Lu et al., 2002; Sinha et al., 2014). In addition to the beneficial effects of MR, this diet also reduces the body size of mice and rats (Ables et al., 2012; Huang et al., 2014).

To our knowledge, there are only two previous studies that investigated the effects of MR in bones of young growing male mice and rats (Ables et al., 2012; Sinha et al., 2014). We previously reported that mice provided a high-fat methionine-restricted diet (HFD-MR) exhibited growth restriction compared to their HFD-CF (control-fed) counterparts, which could be due to increased collagen degradation (Ables et al., 2012). The HFD-MR mice had smaller femurs with reduced bone mass density (BMD) and bone mineral content (BMC) compared to HFD-CF mice (Ables et al., 2012). Huang et al. reported that MR rats were smaller; had reduced bone mass compared to CF rats, as determined by micro-computed tomography (μCT); and had decreased extrinsic strength, as measured by a 3-point bending test (Huang et al., 2014). However, bones from MR rats had higher intrinsic biomaterial strength and toughness compared to CF rats (Huang et al., 2014). These studies suggest that MR affects overall bone development in rodents.

A salient characteristic of MR is its ability to induce hyperhomocysteinemia (HHcy) in rodents due to decreased cystathionine β-synthase (CBS) activity (Ables et al., 2015; Elshorbagy et al., 2010). Interestingly,

Abbreviations: BMC, bone mineral content; BS, bone surface; BV, bone volume; CF, control-fed; Conn.Dn., connectivity density; CTX-1, C-terminal telopeptide of type 1 collagen; FGF21, fibroblast growth factor-21; HFD, high-fat diet; HHCy, hyperhomocysteinemia; IDI, indentation depth increase; IGF-1, insulin-like growth factor-1; I_{max}, maximal MOI; I_{min}, minimal MOI; LPD, low protein diet; MOI, moment of inertia; MR, methionine restriction; OC, osteocalcin; OPG, osteoprotegerin; P1NP, N-terminal propeptide of type 1 procollagen; pMOI, polar MOI; RANKL, receptor activator for nuclear factor κB ligand; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TV, total volume; vBMD, volumetric bone mass density; μCT, micro-computed tomography.

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HHCy, an independent risk factor for cardiovascular disease, did not alter cardiac function in MR mice (Ables et al., 2015). In addition, Tyagi et al. reported that HHcy in mice reduced bone mass and affected bone remodeling due to altered bone blood flow (Tyagi et al., 2011). Furthermore, Vijayan et al. reported that HHCy induced alterations in osteoprotegerin/RANKL ratio suggesting increased osteoclast activity which could lead to bone loss (Vijayan et al., 2013). Our current studies, however, focused on the effects of MR in bone metabolism which could be indirectly affected by HHCy.

The potential to translate the benefits of MR into the clinical setting due to its favorable effects with regard to the prevention of diabetes, obesity, and cancer is increasing (Ables et al., 2012; Perrone et al., 2008; Plaisance et al., 2011; Komninou et al., 2006; Sinha et al., 2014; Lu et al., 2003).

To contribute to the overall understanding of MR, we conducted a study on its effects on bone growth and development. Our current study focused on the bones of young and aged mice. Importantly, our studies addressed whether gender is a factor in the effects of MR, which has not been fully explored in the field of bone biology. Finally, we identified a potential molecular mechanism of MR in bones using MC3T3-E1 preosteoblast cell line.

2. Materials and methods

2.1. Animal care

All of the experiments were approved by the Institutional Animal Care and Use Committee of the Orentreich Foundation for the Advancement of Science, Inc. (Permit Number: 0511MB). Male and female C57BL/6J (Stock #000664) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and individually housed in a conventional animal facility maintained at 20 ± 2 °C and a 50 ± 10% relative humidity with a 12 h light: 12 h dark photoperiod. Young males and females were 8 weeks old at the initiation of the experiments and 20 weeks old upon termination. Aged male and female mice (retired breeders from the Jackson Laboratories) were 9 months old at the initiation of the experiments and 12 months old upon termination. Food and water were provided ad libitum. The diet ingredients and feeding protocol have been described previously (Ables et al., 2012, 2015). Briefly, upon arrival, the mice were acclimatized for one week and fed Purina Lab Chow #5001 (St. Louis, MO, USA). Afterwards, they were weight matched and separated into either CF (0.84% methionine w/w) or MR (0.12% methionine w/w) diets consisting of 14% kcal protein, 76% kcal carbohydrate, and 10% kcal fat (Research Diets, New Brunswick, NJ, USA) for 12 weeks. The diet compositions are shown in Supplementary Table 1. Body weight and food consumption were monitored twice weekly. On the day of sacrifice, animals were fasted for 4 h at the beginning of the light cycle to establish a physiological baseline. Mice were sacrificed by CO_2 asphyxiation. Blood was collected from the retro-orbital plexus, and plasma was collected, flash frozen, and stored at −80 °C until analyzed.

2.2. Animal measurements and bone sample preparation

Under light isoflurane anesthesia, length measurements were made from the tip of the nose to the base of the tail of each mouse every 4 weeks for young mice and every 2 weeks for aged mice. After sacrifice, the bones were separated from the soft tissue and processed as described previously (Ables et al., 2012; Huang et al., 2014). Briefly, the tibiae were stored in 70% ethanol for μCT scanning. The femur length was measured using a caliper ruler from the head of the femur to the distal condyle. The bones were then cleaned of soft tissue, wrapped in gauze, immersed in PBS (pH 7.4), and stored in aluminum foil at −80 °C for nanoindentation test. Bones were collected according to different tests, as described below.

2.3. Micro-computed tomography (μCT) analysis

Bone histomorphometry was conducted as described previously (Huang et al., 2014). Briefly, tibiae that were subjected to μCT scanning (SkyScan 1176, SkyScan, Belgium) using the following parameters: Al 0.5-mm filter, 48 kV, 200 μA, 1° rotation step per picture with 2600 ms exposure time, and 9 μm pixel size. Cross-section images (8-bit BMP file) were reconstructed using NRecon (version 1.6.9.4, SkyScan, Belgium) with the following parameters: dynamic range = 0–0.13, smoothing = 2, ring artifact correction = 6, and beam hardening correction (%) = 22. Various densitometry and histomorphometry analyses were performed using CT-Analyzer (version 1.12.0.0; SkyScan) with the gray threshold consistently selected over a range of 50–255. Volumetric bone mass density (vBMD, g/cm^3) and bone mineral content (BMC, mg) measurements were conducted on the whole tibiae, midshaft cortical bones (transverse slices of 1 mm in thickness), and secondary spongiosa of each tibia (transverse slices between 0.5 and 2.5 mm below the lowest point of the growth plate at the distal metaphysis without cortical bone). The following were measured in secondary spongiosa: histomorphometric indices of bone volume (BV, mm^3) and the BV to total volume (TV) ratio (BV/TV, %), bone surface (BS, mm^2), BS to BV ratio (BS/BV, 1/mm), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, mm), connectivity density (Conn.Dn., 1/mm^3), and structure model index (SMI). In addition, a transverse-CT slice was acquired to assess cross-sectional parameters, including the bone area (mm^2) and 3 indices of cross-sectional moment of inertia (MOI): polar MOI (pMOI), maximal MOI (I_{max}), and minimal MOI (I_{min}).

2.4. Bone material testing by nanoindentation

The material level intrinsic properties were assessed using a nanoindentation system (MTS/Agilent XP, Santa Clara, CA, USA) consisting a 60 ± 5° diamond conical indenter with a radius of 2 μm at the tip (Dubois-Ferriere et al., 2014). In brief, femurs were thawed at room temperature, glued to an aluminum stage, and moistened with PBS (pH 7.4) solution for the indentation test. The test was performed on the posterior cortical bone surface at the midshaft of the femur. For each indentation location, two identical trials were performed using the following protocol: ultimate load of 100 milli-Newtons (mN), loading/unloading rate of 1 mN/s, and ultimate-load holding time of 30 s. After 5 min of recovery for the viscoelastic property, a second identical test was conducted on the same location. The indentation hardness (H) and modulus (E) were calculated using the following equations:

$$H = P/A$$

$$E = \frac{1}{2}\cdot\frac{\sqrt{\pi}}{\sqrt{A}}\cdot\frac{dP}{dh}$$

where H is hardness, P is the indentation load, A is the projected contact area, E is the indentation modulus, and $\frac{dP}{dh}$ is the maximal slope of the unloading curve. In addition, the distance between two ultimate load depths and two indentations on the same location was measured as the indentation depth increase (IDI, nm) (Hansma et al., 2008). For each femur sample, the repetitive indentation trial was conducted in two locations at a distance of 0.5 mm from each other.

2.5. Blood biochemical tests

ELISA kits were used to detect the N-terminal propeptide of type 1 procollagen (P1NP), C-terminal telopeptide of type 1 collagen (CTX-1) (Immunodiagnostic Systems, Fountain Hills, AZ), receptor activator for nuclear factor κB ligand (RANKL), leptin, insulin-like growth factor-1