Bone 93 (2016) 104–112

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Reduced femoral bone mass in both diet-induced and genetic hyperlipidemia mice☆

Xiang Chen [a,1], Chunyu Wang [a,1], Kun Zhang [a], Ying Xie [a], Xiao Ji [a], Hui Huang [b], Xijie Yu [a,*]

[a] Laboratory of Endocrinology and Metabolism, Department of Endocrinology, West China Hospital, Sichuan University, 610041, People's Republic of China
[b] Department of Endocrinology, West China Hospital, Sichuan University, 610041, People's Republic of China

ARTICLE INFO

Article history:
Received 16 May 2016
Revised 24 August 2016
Accepted 19 September 2016
Available online 23 September 2016

Keywords:
Hyperlipidemia
Bone metabolism
Sphingosine-1-phosphate
Peroxisome proliferator-activated receptor-γ

ABSTRACT

Growing evidence argues for a relationship between lipid and bone metabolisms with inconsistent conclusions. Sphingosine-1-phosphate (S1P) has been recognized as a suitable candidate for possible link between lipid metabolism and bone metabolism. This study was designed to investigate the effects of hyperlipidemia on bone metabolism using diet-induced and genetic-induced hyperlipidemia animal models and to explore whether S1P is involved. Wild-type mice and low-density lipoprotein receptor gene deficient (*LDLR*$^{-/-}$) mice at age of 8 weeks were placed on either control diet or high-fat diet (HFD) for 12 weeks. Bone structural parameters were determined using microCT. Cross-linked type I collagen (CTx) and S1P levels in plasma were measured by ELISA methods. Bone marrow cells from wild type and *LDLR*$^{-/-}$ mice were induced to differentiate into osteoblasts, osteoclasts and adipocytes respectively. Gene expressions in distal femur metaphyses and cultured cells were studied by qRT-PCR. Moderate hypercholesterolemia was found in HFD-feeding mice; severe hypercholesterolemia and moderate hypertriglyceridemia were present in *LDLR*$^{-/-}$ mice. Femoral trabecular bone mass was reduced in both diet-induced and genetic hyperlipidemia mice. Mice feeding on HFD showed higher CTx levels, and mice with hyperlipidemia had elevated S1P levels. Correlation analysis found a positive correlation between CTx and S1P levels. Lower *Runx2* expression and higher *TRAP* expression were found in both diet-induced and genetic hyperlipidemia mice, indicating decreased osteoblastic functions and increased osteoclastic functions in these mice. Bone marrow cells from *LDLR*$^{-/-}$ mice also showed increased adipogenesis and inhibited osteogenesis accompanied by enhanced *PPARγ* expression. In conclusion, our study found decreased bone mass in both diet-induced and genetic hyperlipidemia mice.

1. Introduction

Previous studies found an increased risk of osteoporosis in subjects with atherosclerotic diseases or metabolic syndrome, suggesting an underlying link may mediate those chronic metabolic diseases. Hyperlipidemia, especially hypercholesterolemia, serving as the most prominent and well-established risk factor for atherosclerosis and a major component of metabolic syndrome, has been suggested to be the possible link. For example, higher risk of hip fracture was found in women after a diagnosis of a cardiovascular disease [1]. In postmenopausal women not taking hormone replacement therapy, total cholesterol (TC) and low density lipoprotein (LDL) levels have been found to be negatively associated with bone mineral density (BMD) at all measured sites [2] . In Japanese postmenopausal women, higher LDL-C levels were associated with increased risks for non-vertebral fractures [3]. Hypercholesterolemia mice also present an osteoporotic phenotype, including increased osteoclasts (OCs), reduced trabecular number and mechanical properties [4]. Statins, the first-line drugs for hypercholesterolemia, show beneficial influence of increasing BMD and reducing fracture risk in humans [5,6]. Statins regulate lipid metabolism exclusively through liver, where they inhibit cholesterol synthesis, leading to increased LDL uptake by the hepatocytes. Therefore, it seems likely that the beneficial effects of statins originate mainly from lipid-lowering, and not from direct effects on bone.

Low-density lipoprotein receptor-knockout (*LDLR*$^{-/-}$) mice, presenting with markedly increased serum TC, LDL-C levels and moderately elevated triglyceride (TG) levels as a result of impaired removal of

Abbreviations: BMD, bone mineral density; BMSC, Bone marrow stromal cell; Col1a1, collagen type 1 alpha-1; CTSK, cathepsin K; CTx, cross-linked type I collagen; HDL, high density lipoprotein; HFD, high-fat diet; LDL, low density lipoprotein; LDLR$^{-/-}$, Low-density lipoprotein receptor-knockout; LRP, low-density lipoprotein receptor-related protein; uCT, microCT; MMP9, matrix metallopeptidase 9; NFATc1, Nuclear factor-activated T cells c1; NHANES, National Health and Nutritional Examination Survey; OCN, osteocalcin; OPPG, osteoporosis-pseudoglioma syndrome; PPARγ, peroxisome proliferator-activated receptor-gamma; RANKL, the receptor activator of nuclear factor-kappa B ligand; Runx2, Runt-related transcription factor 2; S1P, sphingosine-1-phosphate; Tb·N, trabecular number; Tb·Sp, trabecular spacing; Tb·Th, trabecular thickness; TC, total cholesterol; TG, triglyceride; TRAP, resistant acid phosphatase; VF, visceral fat.

☆ Author disclosures: The authors declare that there are no conflicts of interest.

* Corresponding author at: Laboratory of Endocrinology and Metabolism, West China Hospital, Sichuan University, 610041, People's Republic of China.

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bone.2016.09.016
8756-3282/

cholesterol-rich lipoproteins from the plasma compartment, are widely used animal models for studying mechanism underlying hyperlipidemia related diseases such as atherosclerosis, type 2 diabetes and non-alcoholic steatohepatitis [7,8]. Soares et al. found smaller trabeculae, thinner spongy bone and impaired biomechanical properties in *LDLR*$^{-/-}$ mice, rendering these mice were more prone to fracture [9]. Low-density lipoprotein receptor-related protein 5 (LRP5) and LRP6, members of the LDLR family, are co-receptors of Wnt signaling pathway and are all involved in lipid as well as bone metabolism. Human carrying *LRP5* or *LRP6* mutations exhibit hypercholesterolemia and impaired glucose tolerance [10,11]. On the other hand, mice with either heterozygous or homozygous mutations in *LRP5* or heterozygous mutations in *LRP6* show limb defects and decreased BMD [12]. Homozygous loss-of-function mutations in *LRP5* lead to the autosomal recessive osteoporosis-pseudoglioma syndrome (OPPG) in human, presenting with severe osteoporosis and early onset blindness [13]. Furthermore, individuals carrying *LRP5* mutations showed lower BMD and higher prevalence of fractures accompanied by hypercholesterolemia [11]. The co-existence of deficient lipid and bone metabolism in carriers of mutant LDLR-related proteins further highlights their potential reciprocal interactions.

However, some studies got contrary results. Framingham Osteoporosis Study, enrolled 712 women and 450 men, found no long-term effects of cholesterol levels on BMD in women and men [14]. National Health and Nutritional Examination Survey (NHANES) III included 13,592 participants and also revealed no significant relationship between TC, LDL, or high density lipoprotein (HDL) levels and BMD after correcting for possible confounding factors [15]. Okayasu et al. reported increased bone mass in *LDLR*$^{-/-}$ mice due to impaired osteoclast formation as a result of defective osteoclastic cell-cell fusion [16]. Furthermore, previous studies reported different effects of hyperlipidemia on osteoblastic and osteoclastic functions [17–22]. The existence of a possible link between hypercholesterolemia and low BMD in most but not all studies indicates more investigations are needed to clarify their relationship.

Both bone and adipose tissue have been considered to have endocrine functions and are mutually regulated. On the one side, bone derived osteocalcin stimulates adiponectin secretion from adipose tissue, resulting in improved insulin sensitivity [23]. On the other side, adipose derived adipokines, such as leptin and adiponectin, show regulatory effects on bone. It is suggested that leptin inhibits bone formation through the central nervous system [24], while stimulates bone formation via the peripheral pathway [25]. Sphingosine-1-phosphate (S1P), a breakdown product of ceramide metabolism, is involved in the development of metabolic diseases including insulin resistance and type 2 diabetes [26–28]. Interestingly, S1P has been found to regulate bone homeostasis through mobilizing osteoclast precursors into bone marrow cavities, resulting in enhanced bone resorption [29,30]. Thus, S1P represents a suitable candidate for possible link between lipid metabolism and bone metabolism.

Therefore, in this study, we will investigate the effects of hyperlipidemia on bone metabolism and the possible mechanism using two hyperlipidemic animal models, and to explore whether S1P is involved.

2. Material and methods

2.1. Animals and intervention

All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at the West China Hospital, Sichuan University. All efforts were made to minimize animal suffering. 16 wild type mice (male) and 16 homozygous *LDLR* gene knockout mice (male) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Both strains were on C57BL/6 J background and maintained in pathogen-free barrier conditions at animal facility of West China Hospital, Sichuan University. The animals were maintained in ventilated racks at a controlled temperature under a 12-h light/dark cycle, and free access to water and diet.

At age of 8 weeks, mice were randomly divided into four groups with 8 animals each: (1).WC group: wild-type mice, fed on chow diet; (2).WH group: wild-type mice, fed on HFD; (3). LC group: *LDLR*$^{-/-}$ mice, fed on chow diet; and (4). LH group: *LDLR*$^{-/-}$ mice, fed on HFD. Mice were placed on either a chow diet (64% carbohydrate, 10% fat and 26% protein) or a high-fat diet (28% carbohydrate, 60% fat and 12% protein) for 12 weeks. Both diets were bought from Trophic Animal Feed High-Tech Co., Ltd., (Nantong, Jiangsu, China). Mice were weighed weekly.

2.2. Plasma and tissue sampling

At the end of experiment, mice were euthanized with an intraperitoneal injection of sodium pentobarbital. Blood samples were collected and stored at −80 °C. Visceral fat (VF) (including epididymis fat and retroperitoneal fat) were weighed, immediately frozen in liquid nitrogen and stored at −80 °C. The right femora were immersed into 4% paraformaldehyde immediately and were used for detecting bone structural parameters. The left femora were immediately frozen in liquid nitrogen and stored at −80 °C for detecting mRNA expression levels of bone-related genes.

2.3. Blood chemistry

Plasma TG and TC levels were determined using COD-PAP method (Changchun Huili Biotech CO., LTD., Changchun, China). LDL-C was measured using direct method (Changchun Huili Biotech CO., LTD., Changchun, China). Fasting blood glucose was determined using Lifescan One touch glumeter (Johnson & Johnson, USA). Insulin levels were measured using an ultra-sensitive ELISA kit (Crystal Chem Inc., Illinois, USA). C-terminal telopeptide of type I collagen (CTx) and S1P were also measured by ELISA methods (Wuhan USCN Business co., Ltd., Wuhan, China; MyBioSource, San Diego, California, USA).

2.4. MicroCT 80 for bone structural parameters

Bone structural parameters of *LDLR*$^{-/-}$ and wild-type femora were measured using microcomputed tomography (μCT) (MicroCT80, Scanco Medical AG, Bassersdorf, Switzerland), as previously described [31,32]. Briefly, femurs were scanned for microarchitecture in the metaphyseal region of the distal femur at the energy level of 55 keV, and intensity of 177 μA. The distal trabecular scan started about 0.6 mm proximal to the growth plate and extended proximally 1.5 mm. 100 contiguous slices were used for analysis [33]. This instrument provides high-resolution data for trabecular and cortical bone volume, as well as trabecular number (Tb·N), trabecular thickness (Tb·Th), and trabecular separation (Tb·Sp).

2.5. Real-time qRT-PCR for bone-related gene expression

Femur distal metaphyses were resected and chopped finely with a scalpel as previously described [32]. Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen, Frederick, USA). 1 μg RNA was reversely transcribed into cDNA with PrimeScriptR RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Following reverse transcription, the cDNA (2 μl) was amplified and quantified (Bio—Rad laboratories, Inc., California, USA). The sequence of oligonucleotide primers was listed in Table 1. Each RNA sample was analyzed in triplicate. GAPDH was used as an endogenous control. The relative mRNA expression levels were normalized to the GAPDH in the same sample and analyzed with the $2^{-\Delta\Delta CT}$ method as our previous report [34]. Total RNA was also extracted from visceral fat to study gene expression of *leptin* in the similar way.