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Hepatic lipase is expressed by osteoblasts and modulates bone remodeling in obesity

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ABSTRACT

A number of unexpected molecules were recently identified as products of osteoblasts, linking bone homeostasis to systemic energy metabolism. Here we identify the lipolytic enzyme hepatic lipase (HL, encoded by *Lipc*) as a novel cell-autonomous regulator of osteoblast function. In an unbiased genome-wide expression analysis, we find *Lipc* to be highly induced upon osteoblast differentiation, verified by quantitative Taqman analyses of primary osteoblasts in vitro and of bone samples in vivo. Functionally, loss of HL in vitro leads to increased expression and secretion of osteoprotegerin (OPG), while expression of some osteoblast differentiation makers is impaired. When challenging energy metabolism in a diet-induced obesity (DIO) study, lack of HL leads to a significant increase in bone formation markers and a decrease in bone resorption markers. Accordingly, in the DIO setting, we observe in $Lipc^{-/-}$ animals but not in wild-type controls a significant increase in lumbar vertebral trabecular bone mass and formation rate as well as in femoral trabecular bone mass and cortical thickness. Taken together, we demonstrate that HL expressed by osteoblasts has an impact on osteoblast OPG expression and that lack of HL leads to increased bone mass in DIO. These data provide a novel and completely unexpected molecular link in the complex interplay of osteoblasts and systemic energy metabolism.

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Introduction

Research at the interface of energy metabolism and skeletal biology has been sparked by several findings linking key players of energy metabolism, e.g. leptin as a central regulator of food intake, to obesity and bone mass control [1]. In that vein, studies have highlighted the role of osteoblasts as regulators of whole body energy balance, integrating systemic insulin signaling while providing feedback signals through osteocalcin (OCN) to distant organs such as the pancreas and white adipose tissue [2–5]. One branch of energy metabolism important for the onset of obesity and associated disorders is plasma lipoprotein metabolism. Lipoproteins are major transporters of lipids such as triglycerides, cholesterol and lipophilic vitamins in the bloodstream [6]. Triglyceride-rich lipoproteins (TRL), chylomicrons and very low-density lipoproteins (VLDL), transport dietary and endogenous triglycerides to peripheral organs including adipose tissue, muscle and the heart for storage or combustion [7]. In contrast, the majority of lipids present in low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are cholesterol esters. With regard to bone homeostasis, we and others [8,9] have identified two central players of lipoprotein metabolism, namely apolipoprotein E (apoE) and the giant cell surface receptor LDL receptor-related protein-1 (Lrp1) to be linked to skeletal biology, in particular osteoblast function [10,11]. Interestingly and so far unexplained, apoE-deficient mice display a high-bone mass phenotype with increased bone formation relative to background-matched controls [12]. In humans, apoE is expressed from three different alleles, giving rise to three major isoforms. We have recently shown that mice expressing the human isoforms instead of the endogenous mouse apoE, display differential effects of the isoforms on the skeleton with the human *APOE2* allele being associated with lower bone mass and reduced biomechanical bone stability as compared to *APOE3* and *APOE4* [13]. In addition, we

Abbreviations: ALP, alkaline phosphatase; apoE, apolipoprotein E; BAT, brown adipose tissue; BMD, bone mineral density; CVD, cardiovascular disease; DIO, diet-induced obesity; EpiWAT, epididymal white adipose tissue; FPLC, fast performance liquid chromatography; HDL, high-density lipoproteins; HL, hepatic lipase; LPL, lipoprotein lipase; Lrp1, LDL receptor-related protein-1; µCT, µ-computed tomography; OCN, osteocalcin; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; TRL, triglyceride-rich lipoproteins; VLDL, very low-density lipoproteins; WAT, white adipose tissue.

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have shown that apoE functions as a regulator of bone and energy metabolism in the context of obesity: While in lean mice, apoE-deficiency leads to a high-bone mass phenotype [12], stressing the animals with a high-fat diet (HFD) to induce obesity reduces bone mass and formation rate [14]. In light of reduced weight gain in these animals, apoE appears to be an inverse regulator of bone mass and fat mass in obesity. In addition to TRL, also HDL have been implicated to interact with skeletal cells, protecting osteoblasts from LDL overload-induced cell death [15].

Many other candidate genes have the potential to link bone homeostasis and energy balance. Among these are intraluminal lipases as potent regulators of plasma lipoprotein metabolism. These lipolytic enzymes exhibit specific distinct substrate preferences: Lipoprotein lipase (LPL) hydrolyzes triglycerides, while endothelial lipase (EL) predominantly processes phospholipids; HL has an intermediate affinity for both triglycerides and phospholipids [16]. The vast majority of HL, encoded for by the *Lipc* gene, is produced by hepatocytes and is present in the liver. In the perisinusoidal space of Disse HL converts large, triglyceride-rich HDL_2 to smaller, denser HDL_3 [17] and has been implicated in modulating reverse cholesterol transport [18] and CVD risk [19], a relationship also supported by transgenic mice lacking the mouse *Lipc* gene ($Lipc^{-/-}$) [20]. Findings from a diet-induced obesity (DIO) study in $Lipc^{-/-}$ mice demonstrate an involvement of HL in body weight regulation, decreasing weight gain by decreasing food intake [21]. Much less is known about a potential role of HL in bone and mineral metabolism. One study linked the -514C>T SNP in the *LIPC* gene to bone mineral density (BMD) in postmenopausal Japanese women [22]. This study suggests that HL activity may be linked to bone homeostasis by a yet unidentified mechanism. Surprisingly, in an unbiased micro-array approach to identify genes involved in osteoblast differentiation, we found *Lipc* mRNA to be strongly up-regulated during in vitro differentiation of mouse cultured primary osteoblasts, leading to the hypothesis that HL expression in the skeleton may affect bone metabolism.

Here we investigate the expression and functional relevance of *Lipc* in bone tissue and skeletal cells. Furthermore, we analyze the biological significance of HL for bone homeostasis and obesity development in a DIO study in $Lipc^{-/-}$ and wild-type controls fed a HFD or a fatty acid-matched control diet.

Methods

Mouse treatments

All experiments were approved by Animal Welfare Officers at University Medical Center Hamburg-Eppendorf (UKE). Mice were bred and housed in the UKE animal facility at 22 °C with ad libitum access to water and standard laboratory chow diet (Lasvendi). C57BL/6J $Lipc^{-/-}$ mice were purchased from Jackson (www.jax.org) and only littermates were used for analyses. DIO was induced by feeding a high-fat diet (HFD; Bio-Serv F3282, 35 wt.% lard) ad libitum for 16 weeks beginning at 4 weeks of age [23]. For histomorphometry, we used a calcein 7-day interval double-labeling method [12,14]. Standardized necropsies were performed after 4 h fasting around noon. Mice were anesthetized with a lethal dose of Ketamine/Xylazine, blood was withdrawn by cardiac puncture and animals were perfused with PBS containing 10 U/ml heparin (Ratiopharm). Organs were harvested and immediately conserved in TRIzol (Invitrogen), formalin (see Histology section) or snap-frozen in liquid N_2 and stored at −80 °C. Skeletons were mounted, fixed overnight in 3.7%-formalin and stored in 80%-ethanol. For expression analysis we used 12 week-old male C57BL/6J mice (Jackson).

Primary cell preparation and culture

For primary osteoclasts, femur bone marrow of 12 week-old male mice was harvested by centrifugation (10 s at 8400 *g*) and whole marrow cells were seeded in 6-well plates at a concentration of 5×10^6 per ml in αMEM pH 6.9 (Sigma) containing 10% fetal bovine serum (Invitrogen). Osteoclast differentiation was induced in the mixed marrow cell population without prior removal of adhesive stromal cells by adding MCSF (PEPROTECH) to a final concentration of 20 ng/ml and RANKL to a final concentration of 40 ng/ml (PEPROTECH) for 3 days. Primary osteoblasts were isolated by sequential collagenase Ia (Sigma) and dispase (Roche) digestion of calvaria from 3 day-old mice as described previously [24]. Osteoblast differentiation was induced at 80% confluence in αMEM pH 7.4 (Sigma) containing 10% fetal bovine serum (PERBIO), 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma). At days 6, 15 and 21 of differentiation medium was collected. At day 21 cells were fixed in 4% PFA (Merck) or directly harvested in TRIzol for RNA isolation (see below). Marrow osteoblasts were prepared as described above for osteoclasts except that we used αMEM pH 7.4 (Sigma) containing 10% fetal bovine serum (PERBIO), 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma) to initiate differentiation after 3 days of seeding. Primary hepatocytes were harvested from wild-type mice as described [25]. Cells were seeded in DMEM containing 10% FCS (Invitrogen) to a density of 200,000 cells per well in collagen-coated 12-well plates (Nunc) and harvested in TRIzol 24 h after seeding.

Plasma and urine parameters

Plasma triglycerides and cholesterol were determined using commercial kits (Roche) that were adapted to microtiter plates. For fast performance liquid chromatography (FPLC) pooled plasma was separated using S6-superose columns (GE Healthcare) and lipid levels were analyzed in each fraction as described above. Bone turnover markers were measured according to the manufacturers' instructions: Alkaline phosphatase activity was determined using the NPP method (Sigma), OPG and RANKL were determined by ELISA (R&D), OCN was analyzed by an immunoradiometric assay (Immutopic) and DPD/creatinine were determined by ELISA (Quidel). Blood glucose levels were measured using AccuCheck Aviva sticks (Roche). Plasma adiponectin (R&D) and leptin (R&D) concentrations were determined by ELISA according to the manufacturers' instructions.

Expression analysis

Cells or tissues in TRIzol® (Invitrogen) were disrupted using a TissueLyser (Qiagen). Total RNA was isolated using NucleoSpin RNA II kit (Macherey & Nagel). Complementary DNA was synthesized using SuperScript® III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed on a 7900HT sequence detection system (Applied Biosystems) using TaqMan Assay-on-Demand primers (Applied Biosystems, *Alpl*: Mm01187117_m1; *Bglap*: Mm03413826_mH; *Col1a1*: Mm00801666_g1; *Dmp1*. Mm01208363_m1; *Lipc*: Mm00433975_m1; *Tbp*: Mm00446973_m1; *Tnfrsf11b*: Mm00435452_m1; *Tnfsf11*: Mm013 13943_m1). Gene of interest cycle thresholds (Cts) were normalized to *TATA-box binding protein* (*Tbp*) house keeper levels by the ΔΔCt method and displayed as relative copies per *Tbp* or relative expression normalized to experimental control groups. For genome-wide expression analysis, Affymetrix Murine U74v2 GeneChips containing 36,000 genes and ESTs were used as described by the manufacturer. 10 μg total RNA was used for the cDNA-synthesis. For genome-wide expression analysis the primary osteoblasts isolated by sequential collagenase digestion were subjected to CD11b microbeads (Miletnyi Biotech) to deplete osteal macrophages as described previously [26]. Cultured osteoblasts were then differentiated for 5 and 12 days in the presence of ascorbic acid and ß-glycerophosphate. RNA was pooled from three individually isolated cultures to perform genome-wide expression analysis: GeneChip microarrays were hybridized with the targets for 16 h at 45 °C, washed and stained using Affymetrix Fluidics Station according to the GeneChip Expression Analysis Technical Manual. Microarrays were scanned with Hewlett-Packard-Agilent GeneChip scanner,

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