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RESEARCH PAPER

Gene expression profile in bone of diabetes-prone BB/OK rats fed a high-fat diet

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Abstract A high-fat diet (HFD) has been recognized as a risk factor for diseases such as dyslipidemia, atherosclerosis, obesity, and osteoporosis. However, studies analyzing gene expression after HFD in bone are rare. That prompted us to analyze the expression of selected genes in bone of 4-week-old diabetes-prone B(io)B(reeding) rats. Two breeding pairs were fed a HFD (+10 % tallow) or were fed a normal diet (ND; Ssniff R-Z) before mating and afterward during pregnancy. After the birth of progeny, parents continued to be given HFD or ND until the progeny was weaned (3 weeks). Thereafter, offspring were weaned and were fed the same food as their parents up to an age of 4 weeks. Body weight was measured at an age of 4 weeks, and subsequently 13 HFD rats and 13 ND rats were killed and the tibial bone was harvested to analyze the expression of 53 genes in bone. All rats fed HFD were significantly heavier than rats fed ND after 3 and 4 weeks. The diet also influenced the expression of genes in bone. There were significant differences in 20 out of 53 genes studied between rats fed HFD compared with rats fed ND. Four out of 20 had a lower and 17 out of 20 genes a higher expression in HFD rats, but differences in gene expression showed obvious differences between males and females. There were only two genes that were similarly different between males and females: *Bmp4* and *Atf4*. Two genes, *Foxg1* and *Npy*, were inversely expressed in males and females. It seems that the gene expression is differently regulated by diet during pregnancy and later in life between males and females. Nevertheless, it cannot be excluded that HFD also acts as an epigenetic factor in the development of offspring in utero.

Keywords Weight gain · Obesity · Osteoporosis · Gene expression · Bone

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Introduction

A high-fat diet (HFD) has been recognized as a risk factor for diseases such as dyslipidemia, atherosclerosis, obesity, and osteoporosis (Hou et al. 1990; Steinberg 1991; Yang et al. 2008; Salem et al.1992; Kopelman 2000; Parhami et al.2000; Chen et al. 2010; Cao 2011). In growing animal models, a HFD or high-energy diet could deleteriously affect bone mineral content, structure, and mechanical properties (Li et al. 1990; Zernicke et al. 1995; Ward et al. 2003). In addition, significant correlations were observed between body composition, adiponectin, and bone parameters in growing mice and rats fed a HFD (Lac et al. 2008; Devlin et al. 2010). Furthermore, it was shown that an atherogenic diet inhibits bone formation by blocking

differentiation of osteoblasts in growing mice, possibly resulting from lipid oxidation products (Parhami et al. 2001). A recent study has suggested that a HFD may induce an increase in bone resorption in mice (Cao et al. 2009, 2010). However, HFD can also have positive effects as shown in the diabetes-prone BioBreeding/OttawaKarlsburg (BB/OK) rat that is an excellent animal model for type 1 diabetes (Yang and Santamaria 2006; Bahr et al. 2011). HFD protects BB/OK rats from developing type 1 diabetes in a sex-specific manner. Furthermore, the expression of lipid-related genes was especially influenced in subcutaneous adipose tissue by a high-fat diet (Bahr et al. 2011). Studies analyzing gene expression after a HFD in bone are rare and mostly done in mice (Xiao et al. 2010, 2011). That prompted us to analyze the expression of selected genes in bone of 4-week-old BB/OK rats, which were fed a HFD ($n = 13$) or a normal diet (ND; $n = 13$) during pregnancy of their mothers and up to an age of 4 weeks. The expression of 53 genes playing a role in bone and lipid metabolism as well as in immunologic reactions was studied in bone (cf. Table 1 in Supplementary Material and M&M).

Materials and methods

Animals

All rats were bred and housed in our own animal facility. They were kept under strict hygienic conditions and had free access to food and acidulated water. All experiments were performed in accordance with the regulations for animal care of the Ministry of Nutrition, Agriculture, and Forestry of the Government of Mecklenburg-Vorpommern (Germany).

Two breeding pairs were fed a high-fat diet (HFD; Ssniff R-Z + 10 % tallow, Soest, Germany) or a normal diet (ND; Ssniff R-Z) 1 week before mating and afterward during pregnancy. After the birth of progeny, parents continued to be given a HFD or ND until the progeny was weaned (3 weeks). At 3 weeks postpartum, offspring were weaned, and the sexes caged separately and fed the same food as their parents up to an age of 4 weeks. Body weight of one complete litter was measured at an age of 4 weeks, and then 13 rats (6M:7F) fed HFD and 13 rats (7M:6F) fed ND were killed with an overdose of anesthesia (Sevofluran, Abbott, Germany) to analyze the gene expression in bone.

RNA isolation

At the time of euthanasia, the tibial bone was harvested from the proximal metaphysis to the tibiofibular junction, excluding all cartilaginous and soft tissue. The tibias were snap frozen in liquid nitrogen and pulverized. Total RNA was extracted with Trizol (Qiagen, Hilden, Germany). Residual DNA was removed by DNase treatment (RNase-Free DNase Set; Qiagen, Hilden, Germany) according to the manufacturer's instructions. A defined amount of purified RNA (1.5 μg) from bone samples was transcribed into cDNA and stored at −20 °C until use, as detailed before (Klöting et al. 2005a, b).

Real-time polymerase chain reaction (qRT-PCR)

Real-time polymerase chain reaction (qRT-PCR) was performed using the ABI PRISM Sequence Detection System 7000 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, using ABI PRISM 7000 SDS Software v1.1. described in detail elsewhere (Klöting et al. 2005a). Each quantitative PCR was performed in duplicate.

Target cDNAs were amplified by primer sets as summarized in Table 1 in Supplementary Material and described earlier (Klöting and Klöting 2004; Klöting et al. 2005a, 2006). The rat *18SrRNA* gene (eukaryotic *18SrRNA* endogenous control; FAM Dye/MGB Probe, Applied Biosystems) served as the endogenous reference gene. The melting curve was done to ensure specific amplification.

The standard-curve method was used for relative quantification. For each experimental sample, the amounts of targets and endogenous reference, *18SrRNA*, were determined from the calibration curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value. The relative gene target expression was also normalized to the normal diet-group tissue sample (calibrator). Each of the normalized target values was divided by the calibrator-normalized target value to generate the final relative expression. Final results are expressed as N-fold differences in selected gene expression relative to the *18srRNA* gene and the calibrator.

Statistical analysis

Data are given as mean ± SEM, and differences were assessed by two-way analysis of variance using the statistical analysis system SPSS (SPSS Inc., Chicago, IL, USA). Significant differences are given as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

At first, all rats fed a HFD were significantly heavier than rats fed a ND after weaning and 1 week later, as shown in Fig. 1. The diet also influenced the expression of genes in bone as summarized in Fig. 2. There were significant differences in 20 out of 53 genes studied between rats fed a HFD compared with rats fed a ND. One can differentiate

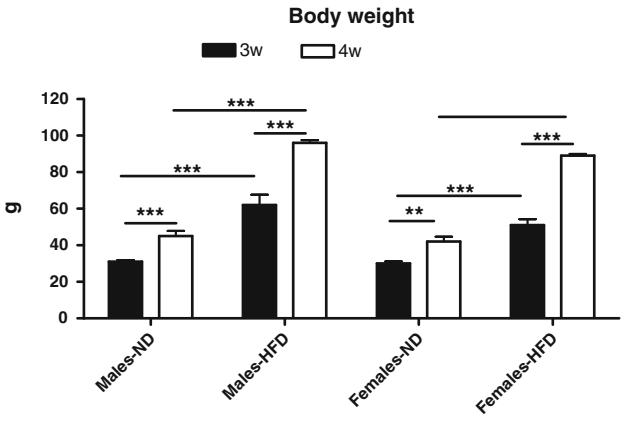

Fig. 1 Body weight at 3 (weaning, *dark bars*) and 4 weeks of age (*light bars*). Data are given as mean ± SD. Significant differences at 0.1 (***) or 1 percent (**) level

between genes that were significantly more highly expressed in ND-fed rats (4/20) or more highly expressed in HFD rats (17/20), but gene expression also showed obvious differences between males and females. Contrary expression patterns between males and females were observed in two genes (*Npy* and *Foxg1*) differing between HFD and ND. In males, the gene expression was significantly increased, whereas in females, the gene expression was decreased in HFD. Another expression behavior was found in *Bmp4* and *Aft4*: both genes were significantly increased in male and female rats fed a HFD. Comparing HFD males and females, there were eight out of 20 genes in males (*Lpr*, *Repin1*, *Dlk1*, *Ccl2*, *Col11a2*, *Mmp9*, *RT1Da*, and *Bmp3*) and eight out of 20 genes in females (*Lep*, *Slc2a4*, *Rnd3*, *Nfkb*, *Ppp3a*, *Vegf*, *Cd1*, and *Ibsp*), which were significantly more highly expressed in only one sex. They were uniquely increased in males and females, respectively. In addition, three genes in males (*Foxg1*, *Mmp9*, and *Bmp3*) and two genes in females (*Rnd3* and *Ibsp*) particularly stand out, because they are several times more highly expressed in HFD than ND rats ($p < 0.001$) and than all the other genes shown in Fig. 2.

Contrary to expectations, no significant differences were found in expression of genes, which are markers of osteoclasts (*Tcirg*), of osteocytes (*Sost*), or of genes regulating osteoclast differentiation like *Tnfsf11* (*Rankl*). Also the expression of osteocalcin (*Bglap*) and insulin receptor (*Insr*) showed no significant differences in bone between BN, DA, and WOKW.

Discussion

Nutrition, including dietary fat, is related to molecular markers of bone remodeling and may contribute to the risk

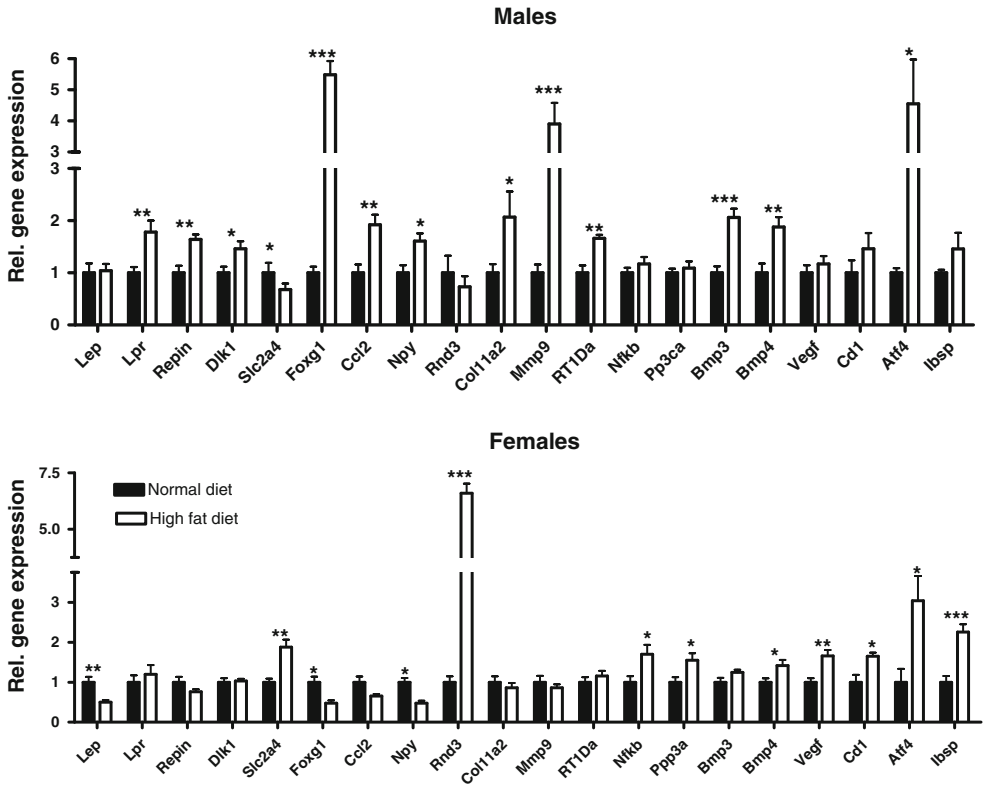

Fig. 2 Relative gene expression in bone of 4 weeks old BBOK rats fed HFD or ND. Data given as mean ± SEM. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$

of bone-related diseases, which is supported by our findings (Watkins et al. 2000; Seibel 2002). Several genes studied were significantly different between HFD and ND rats, a result supporting the findings of Xiao et al. (2010, 2011) analyzing the gene expression between male mice fed HFD or ND. They used a gene microarray system that contained over 22,000 probe sets for 12,960 different genes. Therefore, they found 89 genes that were changed in the HFD group compared with the control group. There were three genes that were also different in our study: *Col1a1*, *Mmp9*, and *Bmp4*. However, the difference between HFD versus ND was not comparable. In addition, they studied only males, so that sex differences could not be found, a phenomenon that was observed in our study. There were only two out of 20 genes that were comparable between males and females: *Bmp4* and *Atf4*. All other genes were differently expressed between males and females.

In contrast to *Bmp4*, *Atf4* belongs to a large family of transcription factors. Its protein binds DNA via their basic regions and dimerize via their leucine zipper domain to form a variety of homo- and heterodimers to regulate gene transcription (De Angelis et al. 2003). It was demonstrated that *Atf4* plays a direct role in regulating osteoclast (OCLs) differentiation and findings suggest that it may be a therapeutic target for treating bone diseases associated with increased OCL activity (Cao et al. 2010). It is also known that *Aft4* influenced the diet-induced obesity. From knockout (KO) mouse studies, it is known that *Atf4* null mice are lean, and they are resistant to age-related and diet-induced obesity. *Atf4* null mice are also hypoglycemic, indicating that *Atf4* regulates mammalian carbohydrate metabolism (Seo et al. 2009; Yoshizawa et al. 2009).

It is noteworthy that *Foxg1* and *Npy* are inversely expressed in males and females. They are more highly expressed in HFD males and lower in HFD females. *Foxg1* encodes a developmental transcription factor with repressor activities (Murphy et al. 1994). The specific function of this gene has not yet been determined. However, it plays a role in the development of the brain and telencephalon and is therefore mainly expressed in the brain, but also in the testes. Therefore, ours is the first study to show that this gene is also expressed in bone in a sex-dependent manner. Mutations in *Foxg1* gene cause the so-called Rett syndrome, which is a severe neurodevelopmental disorder affecting almost exclusively girls (Jacob et al. 2009). In vitro studies have shown a strong expression of *Foxg1* in androgen receptor (AR)-abundant areas of the adult brain, which suggests possible involvement in neuroendocrine regulation. Furthermore, because of the repression of transcription by direct binding to DNA, *Foxg1* may interact with AR in vivo, thereby targeting its repressor function specifically to sex hormone signaling (Obendorf et al. 2007). Therefore, one could speculate that the sex-specific expression of *Foxg1* in bone of HFD BB/OK rats may be caused by the sex-specific action of this gene.

In contrast to *Foxg1*, *Npy* is a neuropeptide that is widely expressed in the central nervous system and influences many physiological processes, such as stress response, food intake, circadian rhythms, and cardiovascular function (Abe et al. 2010). Studies with KO mice have shown that the *Npy* system in the hypothalamus is also implicated in bone remodeling, since *Npy* KO mice have increased bone mineral density (BMD) (Cawley and Yanik 2010). This might be interpreted as a physiological interplay between *Npy* in energy metabolism and bone formation. That is true for our findings in bone; however, this study found sex dependence in bone under a HFD. Edelsbrunner et al. (2009) have shown that female *Npy* null mice are characterized by a significant decrease in water and food intake, but this was not true in males. The difference between males and females found in our study may indicate that the regulation of *Npy* is sex-influenced under a HFD.

Now it is well-accepted that the skeleton is an endocrine organ that, through the secreted molecule osteocalcin (Bgla), favors insulin secretion by insulin-producing β cells and insulin sensitivity in liver, muscle, and adipocytes (Ferron et al. 2008; Fukumoto and Martin 2009; Hinoi et al. 2008; Lee et al. 2007; Lee 2010; Lieben et al. 2009; Schwetz et al. 2012). The protein osteocalcin is produced by osteoblasts and odontoblasts and has been known as a marker of bone turnover (Brown et al. 1984; Ducy 2011). But, we were not able to observe significant differences in *Bgla* expression in bone. Also other genes that are markers of osteoclasts (*Tcirg*), of osteocytes (*Sost*), or of genes regulating osteoclast differentiation like *Tnfsf11* (*Rankl*) or *Insr* showed no significant differences. Therefore, HFD seems not to influence endocrine function of bone in BB/OK rats developing insulin-dependent type 1 diabetes. But, we have not measured serum osteocalcin or have studied the morphology of bone, so that this assumption remains speculative.

Further important findings were the sex dependence of gene expression in bone of males and females. Nearly 50 % of genes demonstrated significantly higher expression under a HFD, but the genes were not comparable between males and females. That could mean that genes of males and females react differently upon fat consumption, or in other words, the digestion of fat is controlled differently depending on sex. This is a plausible consideration, because in mice, it was shown that different protein diets (high vs. normal protein diet) during pregnancy and later in the life of offspring using a HFD versus ND can cause different gene expression between males and females. Sellayah et al. (2008) showed that the mRNA expression

for *Lepr* and for *Npy* genes was significantly lower in male than female offspring. Comparable results were also found in this study for the expression of *Lepr*. It was only significantly increased in HFD males compared to ND males. The values in females were comparable between ND and HFD. Therefore, the gene expression can be differently regulated by diet during pregnancy and later in life in males versus females. However, it cannot be excluded that HFD may also act as an epigenetic factor in the development of offspring in utero. To answer this question, further studies are needed (Gallou-Kabani et al. 2010; Tamashiro and Moran 2010).

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Conflict of interest The authors report no conflicts of interest.

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