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The impact of peripheral serotonin on leptin-brain serotonin axis, bone metabolism and strength in growing rats with experimental chronic kidney disease

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ABSTRACT

Chronic kidney disease (CKD) results in decreased bone strength. Serotonin (5-HT) is one of the critical regulators of bone health, fulfilling distinct functions depending on its synthesis site: brain-derived serotonin (BDS) favors osteoblast proliferation, whereas gut-derived serotonin (GDS) inhibits it. We assessed the role of BDS and peripheral leptin in the regulation of bone metabolism and strength in young rats with 5/6 nephrectomy. BDS synthesis was accelerated during CKD progression. Decreased peripheral leptin in CKD rats was inversely related to BDS content in the hypothalamus, brainstem and frontal cortex. Serotonin in these brain regions affected bone strength and metabolism in the studied animals. The direct effect of circulating leptin on bone was not shown in uremia. At the molecular level, there was an inverse association between elevated GDS and the expression of cAMP responsive element-binding protein (*Creb*) gene in bone of CKD animals. In contrast, increased expression of activating transcription factor 4 (*Atf4*) was shown, which was associated with GDS-dependent transcription factor 1 (*Foxo1*), clock gene - *Cry-1*, cell cycle genes: *c-Myc*, *cyclins*, and osteoblast differentiation genes. These results identified a previously unknown molecular pathway, by which elevated GDS can shift in *Foxo1* target genes from *Creb* to *Atf4*-dependent response, disrupting the leptin-BDS - dependent gene pathway in the bone of uremic rats. Thus, in the condition of CKD the effect of BDS and GDS on bone metabolism and strength can't be distinguished.

1. Introduction

Disturbances in bone metabolism appear between the 3–5 stage of chronic kidney disease (CKD) and result in changes in bone strength [1–2]. Decreases in bone strength manifest clinically as fractures, and it has been recognized as a key component of CKD-related Mineral and Bone Disorder (CKD-MBD) [3].

Bone remodeling is a strictly regulated process controlled by many factors [4]. The neurotransmitter serotonin (5-HT) produced in the brain was discovered to be involved in bone remodeling [5]. This multistep process, named "the central control of bone mass" [6], starts from 5-HT formation from tryptophan (TRP) by the brain-specific enzyme, tryptophan hydroxylase-2 (Tph2). Brain-derived serotonin (BDS), through binding to specific serotonin receptor 5-HT_{2C}, favors bone mass accrual by decreasing sympathetic nervous system (SNS) activity [7–9].

Leptin is an adipocyte-derived hormone that has been known as an inhibitor of bone formation in vivo, although the role of this adipokine in bone remodeling remains controversial. The majority of studies suggest that leptin suppresses bone mass accrual by enhancing sympathetic output to the bone from the hypothalamus, due to inhibition of Tph2 expression and decrease of BDS synthesis [6–9]. The sympathetic tone, by acting through the β_2 adrenergic receptor (β_2-*adr-R*) on the

Abbreviations: CKD, chronic kidney disease; CKD-MBD, CKD-related Mineral and Bone Disorder; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; TRP, tryptopha; BDS, brain-derived serotonin; Tph2, tryptophan hydroxylase-2; SNS, sympathetic nervous system; β_2-*adr-R*, β_2 adrenergic receptor gene; 5-HT_{1B}, serotonin 1B receptor gene; *Rankl*, receptor activator of the NF-κB ligand gene; *Creb*, cAMP responsive element-binding protein gene; *Atf4*, activating transcription factor 4 gene; GDS, gut-derived serotonin; *Per-1*, period-1 gene; *Cry-1*, Cryptochrome-1 gene; *Foxo1*, Forkhead box transcription factor 1 gene; *Runx2*, Runt-related transcription factor 2 gene; *Alp*, alkaline phosphatase gene; *Col1α1*, type I collagen gene; VMA, vanillylmandelic acid; TRACP 5b, tartrate-resistant acid phosphatase form 5b.

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osteoblast surface, may fulfill two functions: it inhibits osteoblast proliferation by using the molecular clock, while it favors bone resorption by increasing the expression of receptor activator of the NF-κB ligand (*Rankl*) gene [5,7–10]. These studies also identified cAMP responsive element-binding protein (*Creb*) and *c-Myc* as the mediators of sympathetic inhibition of osteoblast proliferation, and activating transcription factor 4 (*Atf4*) as a mediator of osteoclast differentiation [9–10]. Moreover, Kajimura et al. [11] demonstrated that this pathway is influenced by leptin.

However, other studies have found that leptin positively regulates bone formation through direct action on the bone [12–14]. Recently, Turner et al. [14] revealed that peripheral and central leptin do not have opposing actions on osteoblasts and that leptin signaling is essential for normal bone resorption and enhances bone formation.

It is well recognized that bone fragility and fracture risk increase substantially in patients with CKD [15–16]. The possibility of precise determination of structural and compositional skeletal changes that occur during CKD progression is essential to the understanding of the skeletal consequences of uremia. Noninvasive methods used in humans for studying bone health usually involve determination of bone mineral density by dual x-ray absorptiometry and serum/urine bone turnover markers assays [3]. However, these methods provide imprecise information, and biomechanical analyses, which directly measure bone strength, are recommended as procedures better reflecting bone quality than noninvasive methods [17–19].

Recently, we showed that peripheral, gut-derived serotonin (GDS) can affect long bone metabolism and strength in young rats with CKD [20]. The purpose of the present study was to explain, through comprehensive analysis performed on the organ, tissue and molecular levels, the role of the central serotonergic system in the regulation of bone metabolism and strength in the same animal model. We also wanted to establish if peripheral leptin can affect BDS turnover, as well as to explain the direct effect of peripheral leptin on bone biomechanical properties and metabolism. Unexpectedly, the obtained results showed that elevated GDS can disrupt leptin-BDS - dependent gene pathway in the bone of CKD rats and by this way it may modulate the BDS-dependent effect on bone metabolism and biomechanics.

2. Material and methods

2.1. Experimental design, tissue sampling

Forty male, 4 weeks old Wistar rats were used. The CKD rats were established by a two-step 5/6 nephrectomy to induce mild degree uremia as described previously in detail [20]. The brains were removed rapidly, and five brain regions: cerebellum, brainstem, frontal cortex, hypothalamus and striatum were isolated and immediately frozen at −80 °C, according to the earlier procedure [21].

2.2. Determination of TRP, 5-HT and 5-HIAA in brain regions

Frozen brain tissues were weighted and homogenized in 20% trichloroacetic acid (TCA), in a ratio of 1:5, respectively. The homogenates were placed at 4 °C for 30 min, and next the samples were centrifuged at 12,000 rpm for 20 min at 4 °C. After centrifugation the supernatant was filtered (0.45-mm Millipore filter) and stored at −80 °C until assayed. TRP and serotonergic pathway metabolites: 5-HT, 5-HIAA were determined by high-performance liquid chromatography (HPLC-ECD), as has been previously described in details [20].

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantification and quality control of RNA was determined using the *Thermo* Scientific *NanoDrop* 2000 spectrophotometer. High quality of RNA sample was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies). An aliquot of 1 μg of total RNA was reverse transcribed with the RevertAid™ First Stand cDNA Synthesis Kit (Fermentas, Canada) according to manufacturer's instructions.

*q*RT-PCR was performed using the Stratagene Mx3005P QPCR System (Agilent Technologies, USA) with the SG qPCR Master Mix (2×) (EURx, Poland). Each reaction was run in triplicate and contained 2 μl of cDNA template along with 0.3 μM primers in a final reaction volume of 25 μl. Cycling parameters were 95 °C for 10 min to activate DNA polymerase, then 40 cycles of 95 °C for 15 s and 60 °C for 30 s, with a final recording step of 72 °C for 25 s to prevent any primer-dimer formation. PCR reactions were checked by including no-RT-controls, by omission of templates and by both melting curve to ensure only a single product was amplified. Standard curves were generated employing a series of four dilutions of cDNA for each gene. Primers were designed using PRIMER-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) software. Primer sequences for each gene are listed in Supplementary Table S1. All results were normalized to the endogenous reference Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The comparative CT method (ΔΔCT) was used for relative quantification of gene expression [22].

2.4. Left femur biomechanical properties

The biomechanical properties of left femurs were determined on two different bone type: the cortical bone of the femoral diaphysis using three-point bending test and the mixed cortico-trabecular structure of the proximal femur by the bending test of the femoral neck, as has been shown in detail previously [19–20].

2.5. Quantitative analysis of long bones using micro-CT

Dissected tibiae were placed in styrofoam tube covered with stretch foil during scanning to prevent drying. Tibiae were scanned using a 1172 SkyScan microCT desktop scanner (SkyScan, Kontich, Belgium) and subsequently reconstructed and analyzed with the packaged NRecon and CTAn software. The X-ray source was operated at 80 kV/124 μA with a 0.5 mm Al filter. Images were acquired at a 5.44 μm resolution with a 0.4° rotational step. Scans were reconstructed with 20% beam hardening and ring correction factor of 6. From the reconstructed datasets, both a trabecular and cortical volume of interest (VOI) was defined for each tibia. The growth plate cross-section served as an anatomical reference from which the long axis of VOI was defined. The trabecular VOI extended from 150 to 350 slices (0.82–1.90 mm) and the cortical VOI extended from 1500 to 1700 slices (8.16–8.98 mm) relative to the growth plate. Trabecular and cortical VOIs were thresholded at 80 and 90, respectively, on an 8-bit grayscale, and 2D and 3D histomorphometric parameters were obtained [23].

Cortical and trabecular bone densities were calculated with the use of 4 mm diameter bone density phantoms (Bruker-MicroCT BMD calibration phantoms) used to mimic rat tibiae. Bone phantoms with densities 0.25 g/cm^3 and 0.75 g/cm^3 were scanned in an identical manner to the bone samples and were used to calibrate bone density values. Morphometrics and densities were calculated for trabecular and cortical regions.

2.6. Serum biochemistry

Serum leptin levels were determined by mouse and rat leptin ELISA kit, produced by BioVendor, Czech Republic. Serum biochemical parameters and markers of bone turnover were measured as mentioned previously [20]. Urine vanillylmandelic acid (VMA) levels were determined using Rat VMA ELISA kit from Shanghai Sunred Biological Technology Co., Ltd., China.