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RANKL/OPG system regulation by endogenous PTH and PTH1R/ATF4 axis in bone: Implications for bone accrual and strength in growing rats with mild uremia

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ABSTRACT

Osteoprotegerin (OPG), receptor activator of NF-κB ligand (RANKL), and parathyroid hormone (PTH) play a central role in the regulation of bone turnover in chronic kidney disease (CKD), but their influence on bone mineral density (BMD) and strength remains unclear, particularly in children. We studied the clinical significance of OPG and RANKL in relation to PTH, femur weight, BMD, and bone biomechanical properties in growing rats after one month (CKD-1) and three months (CKD-3) of surgically-induced mild CKD. Gene expression of parathyroid hormone 1 receptor (PTH1R) and activating transcription factor 4 (ATF4), major regulators of anabolic PTH response in bone, was also determined. Serum PTH and bone PTH1R/ATF4 expression was elevated in CKD-3 compared with other groups, and it positively correlated with femur weight, BMD, and the biomechanical properties of the femoral diaphysis reflecting cortical bone strength. In contrast, bone RANKL/OPG ratios were decreased in CKD-3 rats compared with other groups, and they were inversely correlated with PTH and the other abovementioned bone parameters. However, the PTH-PTH1R-ATF4 axis exerted an unfavorable effect on the biomechanical properties of the femoral neck. In conclusion, this study showed for the first time an inverse association between serum PTH and the bone RANKL/OPG system in growing rats with mild CKD. A decrease in the RANKL/OPG ratio, associated with PTH-dependent activation of the anabolic PTH1R/ATF4 pathway, seems to be responsible for the unexpected, beneficial effect of PTH on cortical bone accrual and strength. Simultaneously, impaired biomechanical properties of the femoral neck were observed, making this bone site more susceptible to fractures.

1. Introduction

Chronic kidney disease – mineral bone disorder (CKD–MBD) is one of the major clinical complications in patients with chronic kidney disease (CKD). CKD–MDB is a skeletal disorder with long-term consequences, such as growth retardation, low peak bone mass, and a tendency to fracture occurrence in the future [1,2]. The mechanisms through which CKD induces bone disorders are multifactorial and remain not fully explained. Bone remodeling has been suggested to play a fundamental role in the maintenance of skeletal integrity via a balance between the bone formation/resorption process. A pivotal role in this process is played by some glycoproteins: receptor activator of nuclear factor kappa-B (RANK), receptor activator for nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) [3]. These molecules constitute a complex system of mediators involved in the modulation of cell function, differentiation and survival during the bone remodeling

Abbreviations: ATF4, activating transcription factor 4; CKD, chronic kidney disease; CKD–MBD, chronic kidney disease–mineral bone disorder; OPG, osteoprotegerin; PTH, parathyroid hormone; PTH1R, parathyroid hormone 1 receptor; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator for nuclear factor κB ligand; vBMD, volumetric bone mineral density

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process. RANKL promotes osteoclastogenesis and bone resorption by binding to its specific receptor RANK, which is expressed on the surface of both precursors and mature osteoclasts. OPG acts as a soluble decoy receptor for RANKL, preventing its interaction with RANK [4]. Imbalances in the RANKL/OPG ratio or RANK signaling underlie the pathology of many skeletal disorders with enhanced bone loss/formation, or diseases with altered bone remodeling, thus the RANKL/OPG ratio is considered to be a major determinant of bone mass and skeleton integrity [5].

A number of clinical studies reported that circulating OPG levels are elevated in predialysis and hemodialysis patients [6,7], and were reduced after successful renal transplantation [8]. Additionally, elevated OPG levels increased concurrently with CKD progression and were associated with all-cause mortality in patients with CKD [9]. However, the OPG/RANKL system has been mainly studied in adults with CKD, there are only a few studies concerning this system in pediatric CKD patients [10–13]. The dynamic changes in growing bones in childhood make the interpretation of bone-mineral alterations more complicated in comparison with adults [10]. So far, the specific mechanisms of how the RANKL/OPG signaling pathway affects bone metabolism and contributes to the pathogenesis of CKD-MBD remains unclear, particularly in pediatric patients.

The parathyroid hormone (PTH) is a key hormone controlling bone metabolism [14]. Current management of CKD-MBD concentrates on establishing an optimum range for plasma PTH, in order to maintain normal bone turnover and avoid ectopic calcification [15]; however, the optimal PTH target range in CKD is not well determined, especially in the pediatric population [16]. Interestingly, PTH is known to affect osteoblasts and bone with a net catabolic and anabolic effect dependent on the pattern of administration; however, the mechanisms responsible for these differing effects are poorly understood [17]. Most cellular actions of PTH are mediated by the PTH-1 receptor (PTH1R), a G protein- coupled receptor that is expressed in osteoblasts [18]. Binding of PTH to its receptor activates multiple intracellular signaling pathways that ultimately affect cellular behavior, and can alter the gene expression of RANKL and OPG in osteoblasts in both *in vitro* and *in vivo* conditions [19,20].

The aim of our study was to investigate the role of PTH and the RANKL/OPG complex in the context of CKD-MBD in a growing organism. To achieve our target, we generated young CKD rats by 5/6 subtotal nephrectomy and performed measurements of serum PTH and the OPG/RANKL system in serum and bone homogenates. Then, we analyzed the relationship between those parameters and bone mineral density (BMD) and the biomechanical properties of bone. Additionally, to explain the cellular mechanism of PTH action on bone, we examined the gene expression of PTH1R and activating transcription factor 4 (ATF4) – major regulators of the anabolic PTH response in osteoblasts [17,21,22]. And then, we analyzed the interactions between ATF4, PTH1R gene expression, and the aforementioned parameters.

2. Materials and methods

Details about the animals' characteristics, experimental design, serum biochemistry and biomechanical testing procedure were described in the details previously [23]. Briefly, 4-week old Wistar male rats were divided into 2 groups: with chronic kidney disease induced by surgical 5/6 subtotal nephrectomy (CKD, n = 22), and sham-operated (CON, n = 22). After one (CON-1; CKD-1) and three months (CON-3; CKD-3, n = 11 per each group; respectively) of the surgery, the rats were subjected to analysis.

2.1. Femur weight, relative femur weight, and volumetric BMD measurements

After dissection and cleaning of all adherent soft tissues, the left femurs were weighed with an automatic balance (OHAUS®, Nanikon, Switzerland; accuracy to 0.0001 g) and the relative weight of the femur (the bone weight expressed in calculation per body mass) was evaluated. Volumetric BMD (vBMD; g/ml) of the left femur was determined based on Archimedes' principle [24] with the use of an automatic balance equipped with instrumentation for determining the density of solids and liquids (RADWAG AS 60/220R2, Radom, Poland; accuracy to 0.0001 g). The bones were weighed submerged in ultra-pure distilled water and then out of the water. vBMD was automatically calculated according to the formula $vBMD = A/(A - B) - (d_{water} - d_{air}) + d_{air}$, where A is the weight of the femur out of water, B is the weight of the femur submerged in water, d_{water} is the density of the redistilled water at a given temperature, and d_{air} represents air density (0.0012 g/ml). The CV for the vBMD measurements was < 1.3%.

2.2. Immunohistochemistry

The bone sections obtained from the proximal tibiae were prepared as described previously [23]. The sections were deparaffinized in xylenes and hydrated in alcohols. In order to exhibit antigens, the tissue sections were heated in a water bath for 20 min in a citrate buffer (pH = 6.0). Then, they were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase. To reduce non-specific staining, the tissues were incubated with 1% bovine serum in PBS for 20 min. Next, incubation was performed with rabbit polyclonal antibody against mouse OPG or RANKL (Anti-Osteoprotegrin antibody ab73400 and Anti-RANKL antibody ab169966, respectively; Abcam, dilution 1:200) for 30 min at room temperature. The reaction was carried out using polymer detection system (ImmPRESS HRP. Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit, made in Goat, Vector Laboratories). A color reaction for peroxidase was developed with chromogene 3,3′ diaminobenzidine (ImmPACT DAB, Vector Laboratories). Immunohistochemical evaluation was done using a light microscope (Olympus CX40) at a magnification of ×400.

2.3. Preparation of bone tissue homogenates

Immediately after biomechanical testing, the slices of bone tissue, taken from the distal femoral epiphysis (trabecular bone region) and femoral diaphysis (cortical bone region), were weighed, thoroughly rinsed, and homogenized in a cold potassium phosphate buffer (50 mM, pH = 7.4; POCh) using a high-performance homogenizer (Ultra-Turrax T25; IKA, Staufen, Germany) equipped with a stainless-steel dispersing element (S25N-8G; IKA) to receive 10% homogenates. The homogenate was centrifuged at 700 × *g* for 10 min at 4 °C, and then the supernatant was collected and stored at −80 °C.

2.4. OPG and RANKL concentrations in serum and bone homogenates

OPG and soluble RANKL (sRANKL) concentrations were measured in the serum and homogenates of the trabecular and cortical bone region using commercial rat-specific Osteoprotegerin ELISA kit and sRANKL ELISA kit from Immundiagnostik AG. The intra- and inter-assay CVs were < 4.1% and 5% and < 2.7% and 4.4% for sRANKL and OPG, respectively. Bone tissue concentrations of RANKL and OPG were adjusted for protein concentration and the OPG/RANKL ratio was calculated.

2.5. Quantitative-real-time-PCR (QRT-PCR) analysis

Total RNA was isolated from the frozen bone tissues with the Thermo Scientific GeneJET RNA Purification Kit (Thermo Scientific, Lithuania), according to the manufacturer's instructions. Quantification and quality control of ribonucleic acid (RNA) was determined using the Thermo Scientific NanoDrop 2000 spectrophotometer. RNA sample quality was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies). An aliquot of 1 μg of total RNA was reverse transcribed