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Skeletal vascular perfusion is altered in chronic kidney disease

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ABSTRACT

Patients with chronic kidney disease (CKD) are at an alarming risk of cardiovascular disease and fracture-associated mortality. CKD has been shown to have negative effects on vascular reactivity and organ perfusion. Although alterations in bone blood flow are linked to dysregulation of bone remodeling and mass in multiple conditions, changes to skeletal perfusion in the setting of CKD have not been explored. The goal of this study was to establish the effect of CKD on skeletal perfusion in a rat model of CKD. In two experiments with endpoints at 30 and 35 weeks of age, respectively, normal (NL) and Cy/+ (CKD) animals ($n = 6$/group) underwent *in vivo* intra-cardiac fluorescent microsphere injection to assess bone tissue perfusion. These two separate time points aimed to describe skeletal perfusion at 30 and 35 weeks based on previous studies demonstrating significant progression of hyperparthyroid bone disease during this timeframe. CKD animals had blood urea nitrogen (BUN) levels significantly higher than NL at both 30 and 35 weeks. At 30 weeks, perfusion was significantly higher in the femoral cortex (+259%, $p < 0.05$) but not in the tibial cortex (+140%, $p = 0.11$) of CKD animals relative to NL littermates. Isolated tibial marrow perfusion at 30 weeks showed a trend toward being higher (+183%, $p = 0.08$) in CKD. At 35 weeks, perfusion was significantly higher in both the femoral cortex (+173%, $p < 0.05$) and the tibial cortex (+241%, $p < 0.05$) in CKD animals when compared to their normal littermates. Isolated tibial marrow perfusion (−57%, $p < 0.05$) and vertebral body perfusion (−71%, $p < 0.05$) were lower in CKD animals. The current study demonstrates two novel findings regarding bone perfusion in an animal model of high turnover CKD. First, cortical bone perfusion in CKD animals is higher than in normal animals. Second, alterations in bone marrow perfusion differed among the stages of CKD and were distinct from perfusion to the cortical bone. Determining whether these changes in bone perfusion are drivers, propagators, or consequences of skeletal deterioration in CKD will necessitate further work.

1. Introduction

Patients with chronic kidney disease (CKD) have accelerated bone loss, vascular calcification and abnormal biochemistries. Together, these factors contribute to patients being at an alarming risk of cardiovascular disease and fracture-associated mortality (Demer and Tintut, 2010). In CKD patients, the risk of cardiovascular disease is increased 3 to 100-fold (Kundhal and Lok, 2005) and the risk of fracture 4 to 14-fold (Alem et al., 2000) compared to the normal population. These risks rise progressively as kidney function deteriorates. More striking, cardiovascular disease accounts for nearly 60% of deaths in those with CKD (compared to 28% in the normal population); similarly over 60% of CKD patients that sustain a hip fracture die within a year (compared to 20% in the normal population) (Coco and Rush, 2000). These striking statistics emphasize the critical need to better understand the underlying mechanism driving altered cardiovascular and skeletal homeostasis, as well as any potential connection between the two.

Bone is a highly vascularized tissue and bone perfusion plays a crucial role in bone growth (Fleming et al., 2001), fracture repair (Tomlinson and Silva, 2014; Maes et al., 2010; Grundnes and Reikerås, 2009), and bone homeostasis (Carulli et al., 2013; McCarthy, 2006). Disturbances to bone blood flow have been shown to have associated effects on bone health and function (Carulli et al., 2013; Prisby et al., 2008; Colleran et al., 2000; Stabley et al., 2015; Stabley et al., 2013). Conditions that alter bone remodeling (diabetes, disuse, aging, estrogen withdrawal, anabolic drug treatment) have all been associated with changes in bone blood flow (Prisby et al., 2008; Colleran et al., 2000; Prisby et al., 2012; Prisby et al., 2007; Kwon et al., 2010; Bergula et al.,

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1999; Prisby et al., 2013; Prisby and Guignandon, 2011). Moreover, disturbances to bone vasculature, due to any of a number of causes, result in alterations in tissue perfusion (Schipani et al., 2009) and often bone loss (Arnett, 2010). CKD-induced elevations in uremic toxins have long been associated with vascular dysfunction of multiple arterial beds through endothelium-dependent, endothelium-independent and/or vascular remodeling mechanisms (Geenen et al., 2016; Palmer et al., 2011; Dhaun, 2006; Costa-Hong et al., 2009). In the setting of CKD, decreased cardiac output (Bleeker et al., 2006), vascular calcification (Moe and Chen, 2008), and endothelial dysfunction (Malyszko, 2010; Le Brocq et al., 2008; Vettoretti et al., 2006) could all contribute to altered end-organ perfusion. Surprisingly data describing alterations in skeletal vascular perfusion in the setting of CKD are lacking.

The goal of the present study was to test the hypothesis that skeletal perfusion is altered in the setting of CKD. To accomplish this goal, we utilized fluorescent microspheres, which lodge in tissue capillaries in direct proportion to the fraction of cardiac output perfusing the tissue. This technique has been shown to allow measurement of organ perfusion as effectively as radioactive microspheres (Glenny et al., 1993), the experimental gold standard (McCarthy, 2006), and has recently been applied to study skeletal perfusion in rats (Aref et al., 2017).

2. Methods

2.1. Animals

Male Cy/+ rats, Han:SPRD rats ($n = 12$) with autosomal dominant polycystic kidney disease (Moe et al., 2009a), and their unaffected (normal) littermates ($n = 12$) were used for this study. Male heterozygous rats (Cy/+) develop characteristics of CKD around 10 weeks of age that progress to terminal uremia by about 40 weeks. Our laboratory has demonstrated that this animal model recapitulates all three manifestations of CKD-Mineral and Bone Disorder (CKD-MBD) - biochemical abnormalities, extraskeletal calcification, and abnormal bone (Colleran et al., 2000; Prisby et al., 2007)(Moe et al., 2009a). There are many other animal models of the systemic repercussions of kidney disease, but unlike the Cy/+ model, most animal models of CKD are either acute injury or developmental/growth alterations and do not model the effect of the progressive nature of CKD on mineral metabolism. The model utilized in the current study (the Cy/+ rat) avoids this drawback. All animals were fed a casein diet (Purina AIN-76A, Purina Animal Nutrition, Shreevport, LA, USA); 0.53% Ca and 0.56% P from 24 weeks on during the experiment, which has been shown to produce a more consistent kidney disease in this model (Moe et al., 2009a). Blood was collected ~24 h prior to the end of the study for measurement of plasma biochemistries. All procedures were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee prior to study initiation.

2.2. Experiments

CKD animals and their normal littermates were used in two separate studies, designed to assess alterations in two distinct time points along the progression of disease in the Cy/+ model:

2.2.1. Experiment 1–30 week time point (~25% normal kidney function)

Normal (NL) and Cy/+ (CKD) animals ($n = 6$/group) were assessed for serum biochemistries ~ one day before undergoing *in vivo* microsphere injection to assess bone tissue perfusion.

2.2.2. Experiment 2–35 week time point (~15% normal kidney function)

Normal (NL) and Cy/+ (CKD) animals ($n = 6$/group) were assessed for serum biochemistries ~ one day before undergoing *in vivo* microsphere injection to assess bone tissue perfusion.

These two separate experiments aimed to describe skeletal perfusion at 30 and 35 weeks were designed based on previous work demonstrating significant progression of skeletal disease in this timeframe (Newman et al., 2014; Moe et al., 2014). While elevations in blood urea nitrogen (BUN) are noted by 25 weeks, progressive hyperphospatemia, hyperparathyroidism, and skeletal abnormalities become evident by 30 weeks. Between 30 and 35 weeks there is marked progression of all of the end organ manifestations of CKD-MBD, including left ventricular hypertrophy, cardiac and vascular calcification, and severe high turnover bone disease evident by severe cortical porosity, high turnover and compromised mechanical properties (Newman et al., 2014; Moe et al., 2014; Hsueh et al., 2014; Moe et al., 2009b).

2.3. Bone perfusion measurement

Microsphere injection was performed as previously described (Aref et al., 2017). Briefly, under isoflurane anesthesia, polystyrene red fluorescent (580/605), 15 μm microspheres (FluoSpheres, ThermoFisher) were injected into the apex of the beating left ventricle after opening the chest cavity. The spheres were allowed to circulate for 60 s before the animal was euthanized by cardiac dissection. A total of 5.0×10^6 spheres/kg were injected, a number sufficient to assess perfusion in skeletal tissue (Aref et al., 2017).

Tibiae, femora, humeri, vertebrae (L4 body), kidneys and testes were collected and weighed. Testes were used as a positive control for assessing adequacy of microsphere delivery within each animal. Microsphere mixing and injection was considered adequate for an animal when right and left testicle perfusions were within 25% of each other. On the basis of this criterion, no animals were excluded from the study. Femur samples were divided into proximal, middle (diaphysis), and distal segments as previously described (Colleran et al., 2000), and weighed separately. Right femoral diaphysis marrow was left intact in bone while left femoral diaphysis marrow was thoroughly flushed and femoral cortex was weighed. Marrow was extracted from the tibial diaphysis by centrifugation; both marrow and tibial cortex were weighed. Marrow was left intact in the remainder of all specimens.

Bone samples were placed in individual amber vials with 15 mL of Cal-Ex Decalcifier solution. After 7 days, decalcified bone samples were placed in 10% ethanolic postassium hydroxide (KOH) for degradation. Soft tissue samples (kidney and testes) were placed in KOH directly. After 24 h of degradation, samples were vortexed to complete the degradation process and then filtered through polyamide mesh filters (5 μm pore size). 1 mL of Cellosolve acetate (2-ethoxyethyl acetate, 98%, Sigma) was added to each of the filtered samples to dissolve the microspheres and expose the fluorescence. The 24 h KOH degradation step differed from the original protocol (Aref et al., 2017), where samples were degraded in KOH for 48 h. This slight alteration was made based on developmental work in our lab showing 24 h was sufficient for degradation with longer durations causing progressive decline in fluorescence.

All fluorescence measurements were made using the SpectraMax i3x microplate reader (Molecular Devices, CA). Three 100 μL aliquots from each sample were placed in a 96-well V-bottom polypropylene microplate for fluorescence quantification. The readings from the three aliquots were averaged to produce a single fluorescence measurement per sample. Red fluorescence was measured using an excitation of 580 nm and an emission of 620 nm. Standard curves of serial dilutions with known amounts of microspheres were generated on the day of analysis. Fluorescent measurements of samples found to be outside the standard curve (kidneys) were serially diluted and measured in order to detect any potential quenching effects. All data is presented as tissue fluorescence density (TFD) with units of Arbitrary Units per gram of tissue (AU/g) and scaled by 10^6.

2.4. Biochemistries

Blood plasma was analyzed for blood urea nitrogen (BUN) and calcium using colorimetric assays (BioAssy System, DIUR-100). Intact