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Uric acid suppresses 1 alpha hydroxylase *in vitro* and *in vivo*

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ABSTRACT

Objective. Patients with gout have lower calcitriol levels that improve when uric acid is lowered. The mechanism of these observations is unknown. We hypothesized that uric acid inhibits 1-αhydroxylase.

Materials and methods. *In vivo*, Sprague Dawley rats were randomized to control (n = 5), allantoxanamide (n = 8), febuxostat (n = 5), or allantoxanamide + febuxostat (n = 7). Vitamin D, PTH, and 1-αhydroxylase protein were evaluated. In order to directly evaluate the effect of uric acid on 1-αhydroxylase, we conducted a series of dose response and time course experiments *in vitro*. Nuclear factor κ-B (NFκB) was inhibited pharmacologically. Finally, to evaluate the potential implications of these findings in humans, the association between uric acid and PTH in humans was evaluated in a cross-sectional analysis of data from the NHANES (2003–2006); n = 9773.

Results. $1,25(OH)_2D$ and 1-αhydroxylase protein were reduced in hyperuricemic rats and improved with febuxostat treatment. Uric acid suppressed 1-αhydroxylase protein and mRNA expression in proximal tubular cells. This was prevented by NFκB inhibition. In humans, for every 1 mg/dL increase in uric acid, the adjusted odds ratio for an elevated PTH (>65 pg/mL) was 1.21 (95% C.I. 1.14, 1.28; P < 0.0001), 1.15 (95% C.I. 1.08, 1.22; P < 0.0001), and 1.16 (95% C.I. 1.03, 1.31; P = 0.02) for all subjects, subjects with estimated GFR ≥60, and subjects with estimated GFR <60 mL/min/1.73 m^2 respectively.

Conclusion. Hyperuricemia suppresses 1-αhydroxylase leading to lower $1,25(OH)_2D$ and higher PTH in rats. Our results suggest this is mediated by NFκB. The association between uric acid and PTH in NHANES suggests potential implications for human disease.

1. Introduction

Chronic kidney disease (CKD) is associated with significant morbidity and mortality [1]. In addition to well-known risk factors such as hypertension and diabetes, several "non-traditional" risk factors may contribute to the higher risk of death in patients with CKD compared to the general population [2–6]. Inflammation and CKD mineral and bone disorder

Abbreviations: CKD, chronic kidney disease; MBD, Mineral and bone disorder; $1,25(OH)_2D$, 1,25 dihydroxy-vitamin D; 25(OH)D, 25 hydroxyvitamin D; PTH, parathyroid hormone; IQR, inter-quartile range; NHANES, National Health and Nutrition Examination Survey; MDRD, Modification of Diet in Renal Disease; GFR, glomerular filtration rate; BMI, body mass index; ATX, allantoxanamide; FBXT, febuxostat; NFκB, nuclear factor κ- B.

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(MBD) are two risk factors that have taken center stage in CKD patients [7,8]. In particular, the progressive decline in 1,25 dihydroxy-vitamin D ($1,25(OH)_2D$) levels that occurs as glomerular filtration rate (GFR) falls has been associated with increased activity of the renin angiotensin system [9], insulin resistance [10], and progressive inflammation [11,12]. In turn, inflammation itself may suppress vitamin D activation thus contributing to the osteopenia seen in inflammatory diseases such as ankylosing spondylitis [13].

Vitamin D, provided in the diet or generated in the skin by ultraviolet light, is normally converted to 25 hydroxyvitamin D (25(OH)D) in the liver. 25(OH)D is then further hydroxylated by a rate-limiting step to $1,25(OH)_2D$ by the enzyme 1-α hydroxylase (CYP27B1); present primarily in proximal tubular cells in the kidney [14]. A decrease in $1,25(OH)_2D$ secondary to reduced nephron mass and/or 1-α hydroxylase enzyme activity contributes to the development of secondary hyperparathyroidism in patients with CKD [15].

The potential for a direct relationship between hyperuricemia and vitamin D metabolism was initially raised more than 20 years ago [16,17], and was subsequently explored by Takahashi et al. [18], where patients with gout were found to have lower $1,25(OH)_2D$ levels than normal controls. The same group showed that treatment of hyperuricemia increases $1,25(OH)_2D$ levels with no change in 25(OH)D [19] suggesting that hyperuricemia may have a suppressive effect on 1-α hydroxylase activity. The mechanism underlying such observations remains unknown. We hypothesized that high uric acid levels reduce $1,25(OH)_2D$ levels by suppressing 1-α hydroxylase and, since low $1,25(OH)_2D$ levels can stimulate parathyroid hormone (PTH), that high uric acid levels should be associated with elevated PTH levels. To test this, we also conducted studies *in vivo* where we utilized an animal model of acute hyperuricemia and *in vitro* using human proximal tubular cells. Furthermore, we evaluated the relation between high uric and elevated PTH levels utilizing data from the National Health and Nutrition Examination Survey (NHANES 2003–2006), a large cross sectional sample of the US population.

2. Methods

2.1. Animal Study

Male Sprague–Dawley rats ($n = 25$) weighing 150 g (Charles Rivers Laboratories, Wilmington, MA) were randomly assigned to one of 4 groups: control (n = 5), allantoxanamide at a dose of 150 mg/kg intraperitoneally (n = 8), febuxostat at a dose of 30 mg/kg by gavage (n = 5), or allantoxanamide and febuxostat (n = 7). Allantoxanamide is a uricase inhibitor that has been shown to acutely raise serum uric acid when injected intraperitoneally in rats [20] at a dose of 100–200 mg/kg [20,21]. Febuxostat is a non-purine selective inhibitor of xanthine oxidase. Contrary to allopurinol, febuxostat does not inhibit other enzymes in purine and pyrimidine metabolism pathways [22], yet has a more potent uric acid lowering effect than allopurinol *in vitro* and *in vivo* [23]. Considering the potent effect of allantoxanamide, febuxostat was administered 1 h prior to allantoxanamide and at a high dose to ensure the uric acid lowering effect. After 24 h, blood samples were obtained and all animals were sacrificed under light anesthesia. Kidneys were immediately fixed in Methyl-Carnoy's solution. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado Denver.

2.2. Biochemical data

Serum chemistries including uric acid, creatinine, calcium, and phosphorus were measured by autoanalyzer (VetAce machine; Alpha Wasserman, West Caldwell, NJ). As previously published by other groups, the ratio of $1,25(OH)_2D$ to 25(OH)D was used as an indirect assessment of 1-α hydroxylase activity [24]. $1,25(OH)_2D$ and 25(OH)D levels were measured by Enzyme-linked ImmunoAssay (ELISA) kits (Immunodiagnostic Systems). The reported intra-assay CV% for 25(OH)D ranges between 5.3% and 6.7% and the interassay CV% ranges from 4.6% to 8.7%. For $1,25(OH)_2D$, the intra-assay CV% ranges between 9.3% and 10.7% and the interassay CV% ranges from 17.1% to 19.7%. Similarly, intact PTH was measured by ELISA (Immunotopics, Inc.).

2.3. Renal histology and Immunofluorescence

Kidneys were fixed in paraffin, sectioned (2 μm thickness), and stained by Periodic Acid- Schiff (PAS) for histological analysis. For immunofluorescence, heat-induced epitope retrieval was achieved in antigen retrieval citrate solution (BioGenex, San Ramon, CA) for 1-α hydroxylase. After rinsing the sections in PBS, they were blocked in 1% normal goat serum for 1 h at room temperature. A rabbit polyclonal anti-rat 1-α hydroxylase antibody (Santa Cruz, CA) was used as primary antibody, and Alexa fluor 568 conjugated goat polyclonal anti-rabit antibody (Invitrogen, Carlsbad, CA) as secondary antibody. Images were analyzed using Axio Vision image analyzer (Carl Zeiss, Thornwood, NY) at 20× and 40×. The same settings of the microscope were applied to all the images being compared. Nonspecific staining with secondary antibody was negligible.

2.4. Immunoblotting

Whole kidney or stimulated HK2 cells were lysed in lysis buffer (20 mmol/L Tris–HCl [pH 8.0], 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 25% Glycerol, and 0.5 mmol/L PMSF). Nuclear and cytosolic proteins were extracted with Biovision extraction kit (Mountain View, CA). Equal amounts of protein were resuspended in SDS sample buffer, boiled for 5 min, and analyzed on 4% to 20% SDS-PAGE gels. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-ECL; Amersham, Piscataway, NJ) and probed with the following antibodies: 1-α hydroxylase and 24 hydroxylase (Santa Cruz, Santa Cruz, CA), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The antigen–antibody complexes were detected by the ECL protocol using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG as secondary antibody. The immunoblots shown are representative of the animal groups.