Dahl Salt-Sensitive Rats Excrete 25-Hydroxyvitamin D into Urine\textsuperscript{1,2}

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ABSTRACT The plasma 25-hydroxyvitamin D concentration of Dahl salt-sensitive rats (S) is markedly decreased in response to high sodium chloride (salt) intake. We tested the hypothesis that urinary excretion is a mechanism for the decrease. Female S rats excreted 0.26 \pm 0.04 nmol 25-hydroxyvitamin D/24 h at wk 2 of high salt (80 g/kg) intake, five times that of female salt-resistant (R) rats at wk 2 of high salt intake and nine times that of S rats at wk 2 of low salt (3 g/kg) intake. The 25-hydroxyvitamin D binding activity in 24-h urine of S rats was 79 \pm 11 pmol/h at wk 2 of high salt intake, two times that in urine of S rats at wk 2 of low salt intake and > 35 times that in urine of R rats at wk 2 of low or high salt intake. We conclude that markedly decreased plasma 25-hydroxyvitamin D concentrations of S rats during high salt intake result in part from excretion of protein-bound 25-hydroxyvitamin D. Low plasma 25-hydroxyvitamin D concentrations in humans may also result in part from salt sensitivity, which is prevalent in > 50\% of the United States hypertensive population. J. Nutr. 133: 187–190, 2003.

KEY WORDS: • salt sensitivity • vitamin D • Dahl rats • sodium chloride

The liver metabolite of vitamin D, 25-hydroxyvitamin D (25-OHD),\textsuperscript{4} is the precursor to the hormone, 1,25-dihydroxyvitamin D [1,25-(OH)\textsubscript{2}D], which is synthesized in the kidney. Plasma 25-OHD concentrations are lower, and 1,25-(OH)\textsubscript{2}D higher, in elderly hypertensive females with low plasma renin activity, characteristic of salt-induced hypertension, compared with normotensive elderly and young females (1). African-Americans, who have a higher rate of hypertension and salt-sensitivity than Caucasian Americans (2,3), have been demonstrated to have significantly lower mean plasma 25-OHD concentrations than Caucasian Americans in several studies involving both men and women (4–10) and in a report (11) based on the Third National Health and Nutrition Examination Survey (NHANES III).

A connection between the vitamin D endocrine system and salt-induced hypertension has been shown for the Dahl salt-sensitive (S) rat, a widely studied genetic model of salt sensitivity (12,13). High salt intake significantly decreases the plasma 25-OHD concentration of S rats (14–18). Blood pressure was shown to be directly correlated (r = 0.97) and plasma 25-OHD concentration indirectly correlated (r = −0.98) with the number of days that S rats were fed a high salt diet (14). An inverse correlation (r = −0.99) between blood pressure and plasma 25-OHD concentration was shown for S rats (14). The decrease in plasma 25-OHD concentration during high salt intake was preceded by a decrease in 25-OHD content in the kidneys of S rats. The 25-hydroxyvitamin D content in the kidneys of S rats at d 2 of high salt intake was 81\% of that in the kidneys of S rats at d 2 of low salt intake (17).

Attempts to increase plasma 25-OHD concentration, by administering 25-hydroxycholecalciferol (25-OH\textsubscript{D\textsubscript{3}}) via osmotic pumps, were successful in S rats fed a low, but not a high salt diet. This suggested increased clearance or accelerated metabolism of 25-OH\textsubscript{D\textsubscript{3}} during high salt intake (18). We report here on the urinary excretion of 25-OH\textsubscript{D\textsubscript{3}} by S rats.

MATERIALS AND METHODS

Animals and diets. Protocols involving animals were previously approved by the Animal Care and Use Committee at Morehouse School of Medicine. Guidelines followed were those of the Public Health Service and the revised animal welfare act as regulated by USDA.

Dahl salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) female rats (4–5 wk old, 100–110 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). The rats were allowed free access to water and a nonpurified diet (Purina, LabDiet 5001, PMI Nutrition International, Brentwood, MO). They were housed in a room with a 12-h light:dark cycle and, after 1 wk of acclimation, were divided into groups of six rats each and fed either a low (3 g/kg) or high (80 g/kg) salt (sodium chloride) diet\textsuperscript{5} for 2 wk. The rats were placed in metabolic cages at baseline and on d 7 of each week for 24-h urine collection. Urine was stored at −80°C. At the termination of the dietary treatments, rats

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\textsuperscript{4} Abbreviations used: DBP, vitamin D binding protein; HS, 80 g/kg sodium chloride diet; LS, 3 g/kg sodium chloride diet; NHANES III, Third National Health and Nutrition Examination Survey; 1,25-(OH)\textsubscript{2}D, 1,25-dihydroxyvitamin D; 25-OHD, 25-hydroxyvitamin D; 25-OHD\textsubscript{3}, 25-hydroxycholecalciferol; R, salt-resistant; S, salt-sensitive.

\textsuperscript{5} Supplied by Harlan Teklad (Madison, WI). Low salt, TD 92101 (g/kg diet): corn, yellow, ground, 311.5; soybean meal, 190.0; corn gluten meal, 50.0; corn oil, 33.0; sodium chloride, 3.0. High salt, TD 92012 (g/kg diet): corn, yellow, ground, 214.7; soybean meal, 201.0; corn gluten meal, 56.0; corn oil, 33.8; sodium chloride, 80.0. Identical components (g/kg diet): wheat, hard, ground, 350.0; alfalfa meal, 30.0; dicalcium phosphate, 14.0; calcium carbonate, 12.0; mineral mix (TD 80318), 1.5; vitamin mix (TD 81125), 3.0; \textalpha;-methionine, 1.0; lysine - HCl, 1.0; ethoxyquin (antioxidant), 0.01.
were anesthetized (ketamine/xylazine, 44:10 mg/kg body) and blood was drawn by heart puncture into heparinized tubes. Plasma was obtained by centrifuging the blood at 1500 \times g for 10 min. Rats were killed by sodium pentobarbital overdose.

**Materials.** The 25-OHD\textsubscript{3} standard was purchased from ICN (Costa Mesa, CA). The concentration of 25-OHD\textsubscript{3} dissolved in ethanol was determined by ultraviolet spectroscopy at 265 nm using a Lambda 3B spectrophotometer (Perkin-Elmer, Norwalk, CT) and a molar absorption coefficient of 18,200 mol/L \textsuperscript{-1} cm \textsuperscript{-1}. Organic solvents were analytical or HPLC grade.

**Measurement of 25-OHD concentration.** Radiolabeled 25-OHD\textsubscript{3}, 25-hydroxy[\textsuperscript{26(27)}-methyl\textsuperscript{3}H]cholecalciferol (Amersham, Arlington Heights, IL), was purified on a 0.46 × 25 cm Zorbax-Sil HPLC column (Agilent Technologies, Wilmington, DE) with dichloromethane/isopropanol (95:5, v/v) at a flow rate of 1 mL/min or on a Zorbax-CN column (Agilent Technologies) with hexane/isopropanol/methanol (94:5:1.5, v/v/v) at a flow rate of 1.3 mL/min (19). Synthetic 25-OHD\textsubscript{3} served as the standard.

A 25-OHD fraction was isolated from 3 mL urine by dichloromethane/methanol liquid-liquid extraction (14) followed by solid-phase separation on silica cartridges by the method of Reinhardt and Hollis (20). The fraction was purified by the HPLC methods described above. Radiolabeled 25-OHD\textsubscript{3} (800 dpm) was added to the urine samples before purification to determine recovery. Protein binding assay kits, containing rat vitamin D binding protein (Nichols Institute Diagnostics, San Juan Capistrano, CA), were used to assay the 25-OHD peaks. Standard curves were constructed using 25-OHD\textsubscript{3} (0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5 ng/tube). The limit of detection of the assay, as designed, was 0.16 nmol/L for urine. The concentration of 25-OHD\textsubscript{3} (nmol/L) was multiplied by the 24-h urine volume to obtain nmol/24 h.

**Measurement of 25-OHD binding activity.** Urine was tested for 25-OHD\textsubscript{3} binding to 25-OHD\textsubscript{3} by modiﬁcation of the method of Reinhardt and Hollis (20) and the assay procedure for 25-OHD of Nichols Institute Diagnostics. Urine samples were diluted with 0.1 mol/L boric acid buffer, pH 8.6 (5–10 fold dilution). Aliquots of the diluted urine samples (0.5 mL) were incubated with radiolabeled 25-OHD\textsubscript{3} (0.58 nmol/L, 10,000 dpm) in the presence of ethanol or 200-fold molar excess unlabeled 25-OHD\textsubscript{3}. Ligands were added in 10 \mu L ethanol. Incubation was at 4\textdegree C for 2 h. A dextran-coated charcoal preparation (0.2 mL) was added to each incubation mixture and incubation was continued for 20 min at 4\textdegree C (20). The dextran-coated charcoal contained 12 gb charcoal (Sigma Chemical, St. Louis, MO), 1.2 g Dextran T-70 (Pharmacia Fine Chemicals, Piscataway, NJ) and 0.5 g BS-globulin suspended in 0.1 mol/L boric acid, pH 8.6. The incubation mixtures were centrifuged at 1800 \times g for 20 min to separate the bound and free metabolites. The supernatant, which contained the bound metabolite, was decanted into a scintillation vial and counted in Bio-Safe II (Research Products International, Mount Prospect, IL). Duplicate incubations were made for each rat. Binding in the presence of 200-fold excess unlabeled 25-OHD\textsubscript{3} (non-specific binding) was subtracted from total binding to obtain specific binding. Binding activity [pmol/(L \cdot h)] was multiplied by the 24-h urine volume to obtain pmol/h.

**Protein.** Urinary protein was measured by the bicinchoninic acid protein assay kit (Sigma), using an automated plate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA). Protein concentration (mg/L) was multiplied by the 24-h urine volume to obtain mg/24 h.

**Statistical methods.** A mean ± SEM was calculated for each group. Data were evaluated using three-way ANOVA followed by the Tamhane’s T2 test (SPSS, Chicago, IL). Effects tested were S vs. R, low salt (LS) vs. high salt (HS), and time. The Mann-Whitney test was used for comparisons of two groups. Differences were considered significant at $P < 0.05$.

## RESULTS

S rats fed low salt excreted 25-hydroxyvitamin D into urine, whereas excretion by R rats was undetectable (Fig. 1). The level of 25-hydroxyvitamin D excreted by S rats at wk 2 of high salt intake was five times that of R rats at wk 2 of high salt intake and nine times that of S rats at wk 2 of low salt intake. Because of a higher level of lipid, necessitating additional HPLC chromatography, baseline urine was not assayed for 25-OHD. The calculated 25-hydroxyvitamin D binding activity in the 24-h urine of S rats (Fig. 2) at wk 2 of high salt intake was twice that in the urine of S rats at wk 2 of low salt intake and >35 times that in the urine of female R rats fed either low or high salt diets at wk 2. Urinary protein (Fig. 3) of S rats was significantly affected by salt and by duration of high salt intake. Urinary protein of S rats was significantly higher than that of R rats.

S rats fed the high salt diet excreted significantly more urine ($P < 0.001$) than R rats fed diet (64 ± 4 vs. 45 ± 4 mL at wk 1; 69 ± 5 vs. 50 ± 2 mL at wk 2), but urine volumes did not differ at either wk 1 or 2 in rats fed low salt diets (range: 9 ± 1 to 11.8 ± 0.3 mL) or at baseline (14 ± 1 vs. 11 ± 1 mL). Urine volumes were greater ($P < 0.001$) in rats fed high salt than in those fed low salt diets.

## DISCUSSION

We have identified both 25-OHD and 25-OHD binding activity in the urine of Dahl S rats that could explain the low

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**FIGURE 1** Urinary excretion of 25-hydroxyvitamin D by Dahl salt-sensitive (S) and salt-resistant (R) female rats fed high (HS) and low salt (LS) diets. Values are means ± SEM, n = 5–6. ND indicates non-detectable. aS vs. R, bLS vs. HS, interaction between salt sensitivity and salt, $P < 0.001$. Effect of time, $P = 0.01$. Interaction between salt and time, $P = 0.03$.

**FIGURE 2** Urinary 25-hydroxyvitamin D binding activity of Dahl salt-sensitive (S) and salt-resistant (R) female rats fed high (HS) and low salt (LS) diets. Values are means ± SEM, n = 5–6 rats, except at baseline ($n = 12$). aS vs. R, bLS vs. HS, interaction between salt sensitivity and salt, $P < 0.001$. Interaction between salt sensitivity and time, $P = 0.01$.
25-OHD concentrations of African-Americans might also be affected by the lower concentrations in a subset of African-Americans with salt-induced hypertension.

It has been estimated that 56% of the Caucasian hypertensive population and 73% of the African-American hypertensive population are salt sensitive (29). Also, the apparent racial differences in response to antihypertensive drugs have been found to be due in part to salt sensitivity (30,31). A multicenter clinical study (32) showed that the salt intake of the U.S. population is high and that lowering dietary salt intake can decrease blood pressure in both African-American and Caucasian subjects. High salt intake significantly affects the calcium endocrine system, markedly decreasing plasma 25-OHD and 24,25(OH)2D concentrations in S rats (14–18), significantly increasing plasma parathyroid hormone concentration in young male S and R rats (33) and young female S rats (16), and significantly increasing plasma 1,25(OH)2D concentration in young female S rats (16). The altered vitamin D endocrine system results in a greater calcicuric response to salt by young female S rats compared with young female R rats (16) and greater calcium excretion by S rats compared with R rats fed a standard diet (16,33–36). If these findings are found to be applicable to human salt sensitivity, the effects of high salt intake on the calcium endocrine system of salt-sensitive individuals would be substantial. Spontaneously hypertensive rats do not have low plasma 25-OHD concentrations (14,37), suggesting that decreased plasma 25-OHD concentration might be specific to salt-induced hypertension. Urinary 25-OHD and 25-OH-binding activity might thus serve as markers of salt sensitivity and aid in its diagnosis and in the prevention and appropriate treatment of salt-induced hypertension.

LITERATURE CITED


FIGURE 3 Protein in the urine of Dahl salt-sensitive (S) and salt-resistant (R) female rats fed high (HS) and low salt (LS) diets. Values are mean ± SEM, n = 5–6 rats, except at baseline (n = 12). *S vs. R, †S vs. HS, P < 0.001. Interaction between salt sensitivity and salt, P = 0.01. Interaction between salt sensitivity and time, P = 0.05.


