Critical role of vitamin D in sulfate homeostasis: regulation of the sodium-sulfate cotransporter by 1,25-dihydroxyvitamin D$_3$

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INORGANIC SULFATE is the fourth most abundant anion in mammalian plasma. As such, sulfate is essential for numerous physiological functions. For instance, sulfate is involved in activation and detoxification of a variety of endogenous and exogenous substances such as xenobiotics, steroids, neurotransmitters, and bile acids (30, 31). Sulfate conjugation is essential for biosynthesis of a large number of structural proteins such as sulfated glycosaminoglycans (a major component of the cartilage), cerebroside sulfate (a constituent of the myelin membranes in the brain), and heparin sulfate (required for anticoagulation) (10, 12, 32). Under sulfation of cartilage proteoglycans has been linked to three types of human inherited osteochondrodysplasia disorders, which are caused by mutations in the diastrophic dysplasia sulfate transporter gene (17, 18, 39, 40). Diastrophic dysplasia sulfate transporter protein transports extracellular sulfate into chondrocytes.

In mammals, sulfate homeostasis is largely regulated by the kidney. The majority of filtered sulfate is reabsorbed in the proximal tubules, and only $\sim$5–20% of the filtered load is excreted into the urine (5, 29). Transcellular transport of sulfate from tubular lumen to blood depends on a sodium-sulfate cotransporter (NaSi-1) in the brush-border membrane (for sulfate entry into the cell) and a sulfate/anion exchanger in the basolateral membrane (for sulfate efflux into the blood), and NaSi-1 is thought to play a regulatory role in this process (4, 27). A similar transcellular transport pathway is also used in the small intestine, mainly the distal ileum, for sulfate absorption.

NaSi-1 was cloned from a rat renal cortex cDNA library by using the Xenopus oocyte expression cloning system (28). The NaSi-1 cDNA is $\sim$2.3 kb long and encodes a 595-amino acid polypeptide with 13 predicted transmembrane domains (4, 27, 28). This cotransporter is predominantly expressed in the kidney cortex and ileum and contains two mRNA species of 2.3 and 2.9 kb. Previous studies show that thyroid hormone, glucocorticoids, and vitamin D can modulate serum sulfate levels, renal sulfate handling, and NaSi-1 expression (14, 35, 41). Vitamin D deficiency in rats leads to lower serum sulfate levels and decreased NaSi-1 expression (14), but sulfate metabolism in vitamin D receptor (VDR)-deficient animals has not been reported. In the present study, we used VDR knockout mice as a model to further investigate the role of vitamin D in sulfate homeostasis. Our results strongly support the notion that vitamin D, through its nuclear receptor, modulates sulfate balance by regulating the expression of renal NaSi-1.

MATERIALS AND METHODS

Animals. Wild-type (WT, or VDR$^{+/+}$) and VDR-null (VDR$^{-/-}$) mice (2–4 mo old), generated from VDR$^{+/+}$ mouse breeding (25), were housed in a pathogen-free barrier facility with a 12:12-h light-dark cycle and fed regular rodent chow. In some experiments, mice were raised on a high-calcium, high-lactose (HCl) diet that contains 2% calcium, 1.5% phosphate, and 20% lactose (Teklad, Madison, WI) to normalize the blood ionized calcium levels in VDR$^{-/-}$ mice (22). To study the effect of 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] on
NaSi-1 expression in vivo, in one experiment, WT mice were injected intraperitoneally with five doses of 1.25(OH)2D3 (30 pmol/mouse) dissolved in 95% propylene glycol-5% ethanol over 3 days. The injections were carried out at 8 AM and 8 PM on the first 2 days, and the last injection was administered at 8 AM on the 3rd day. In another experiment, WT mice were injected with a nonalcemic vitamin D analog, RO-27-5646 (kindly provided by Dr. Uskokovic; dissolved in 95% propylene glycol-5% ethanol), for 7 days at a daily dose of 3 μg/kg body wt. The mice were killed 6 h after the last injection. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of the University of Chicago.

**NaSi-1 cDNA probe.** The NaSi-1 cDNA probe was cloned by RT-PCR using the primers 5’GTGCTCATCTCCCTTTGTTA-TATTG3’ (forward) and 5’GTCAATTITGTGAAGTTTCTG3’ (reverse) based on a published mouse NaSi-1 cDNA sequence (3). Total RNA (5 μg) isolated from WT mouse kidney was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Grand Island, NY). The PCR was carried out as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles. The 1.1-kb PCR product was cloned into pSk(+), and the identity of NaSi-1 cDNA was confirmed by DNA sequencing.

**RNA isolation and Northern blot.** Total RNAs were extracted from kidneys and different segments of the intestine and analyzed by Northern blot as described previously (23). Briefly, tissues were collected immediately after mice were killed and homogenized in TRIzol reagent (Invitrogen). RNA extraction was carried out according to the manufacturer’s instruction. Total RNAs were separated on 1% formaldehyde-agarose gel, transferred onto nylon membranes, and hybridized with 32P-labeled NaSi-1 cDNA probe. The signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). RNA loading was normalized using a 36B4 cDNA probe. NaSi-1 mRNA levels were expressed as relative units based on the ratio of NaSi-1 mRNA to 36B4 mRNA.

**Measurement of serum and urinary sulfate.** Mice were placed in metabolic cages for 24-h collection of urine, which was frozen at −20°C until assay. Urinary creatinine was measured as described previously (24). Inorganic sulfate levels in urine or sera were assayed by a turbidimetric method modified as previously described (26). Briefly, urine was diluted 1:20 with 5% TCA for the assay. To remove proteins in serum samples, 75 μl of sera were mixed with 75 μl of 10% TCA in 0.5-ml tubes. After incubation on ice for 30 min, the samples were centrifuged, and the supernatant was used for the assay. Standards of Na2SO4 at 0.5–2.0 mM (for urine samples) or at 0.125–1.0 mM (for serum samples) were prepared in 5% TCA. For the assay, 50 μl of blanks, standards, or samples were added to a 96-well plate in duplicate, and then 50 μl of 30% glycerol were added. After the contents in the wells were mixed, the plate was covered and incubated for 10 min at room temperature. Then 25 μl of 1% BaCl2 in 10% dextran were added to all wells. After the contents in the wells were mixed, the plate was read within 5 min with a microplate reader (model EL-800, Bio-Tek Instruments, Winooski, VT) set at a wavelength of 600–630 nm. The typical correlation coefficient of the standard curves is >0.99. The precision of this method was verified by assaying rat serum samples. Sulfate concentration in normal mouse or rat serum obtained with this method was highly comparable to that published previously in the literature with use of similar or different analytic methods (7, 20, 36).

**Measurement of proteoglycan content in the skeleton.** Mice skeletal extracts were prepared from Skeletal extracts in dilutions were measured by the dimethylmethylene blue assay as described previously (6). Briefly, skeletal extracts in dilutions were loaded onto a 96-well plate after papain (Sigma, St. Louis, MO) digestion (33). Serial dilutions of purified bovine nasal cartilage aggrecan were used as standards. After addition of the dimethylmethylene blue dye (Serva, Heidelberg, Germany), which binds to the negatively charged sulfate groups mainly on the chondroitin, derma-
...and, keratan sulfate chains, the reaction was measured at 530 and 595 nm, and values were calculated by using the Kineticale Program on the Bio-Tek microplate reader (Denkendorf, Germany).

Measurement of hepatic glutathione content. Livers were dissected from WT and VDR \(^{-/-}\) mice immediately after they were killed, frozen in liquid nitrogen, and stored at \(-80^\circ\)C until use. For the assay, duplicate liver pieces were weighed and homogenized on ice in 5 vol of 5% 5-sulfosalicylic acid by a microcentrifuge Teflon pestle (Bel-Art Products, Pequannock, NJ) attached to a motor-driven drill. After centrifugation for 5 min at 14,000 rpm, the supernatant was diluted 1:40 with 5% 5-sulfosalicylic acid. Total glutathione content was determined by the glutathione reductase-DTNB recycling assay described previously (1) and expressed as micromoles per gram of liver tissue.

Statistical analysis. Values are means ± SD and analyzed with Student’s t-test to assess significance. P ≤ 0.05 was considered statistically significant.

RESULTS

Mice lacking a functional VDR provide an ideal animal model to study the role of vitamin D in sulfate metabolism, because in these mice VDR-mediated vitamin D signaling is completely disrupted. Given the critical role of NaSi-1 in sulfate transport, we first measured the expression of NaSi-1. Northern blot analyses demonstrated a 72% reduction in NaSi-1 mRNA expression in the kidney of VDR \(^{-/-}\) mice compared with WT mice (Fig. 1A). However, NaSi-1 expression in the distal ileum, cecum, and colon of VDR \(^{-/-}\) mice remained unchanged (Fig. 1B and data not shown). These results suggest that vitamin D regulates NaSi-1 expression in a tissue-specific manner, with the kidney being the main vitamin D target in the regulation of sulfate metabolism. Consistent with previous findings (3, 28), two NaSi-1 mRNA species of ~2.9 and 2.3 kb were detected in the kidney and the intestine (Fig. 1).

We then measured urinary sulfate levels in VDR \(^{-/-}\) mice, reasoning that the mutant mice may suffer sulfate wasting because of low renal NaSi-1 expression and, thus, low sulfate reabsorption. Indeed, urinary sulfate levels of VDR \(^{-/-}\) mice were increased by 42% compared with those of WT mice (Fig. 2A); consequently, serum sulfate levels in VDR \(^{-/-}\) mice were decreased by 50% (Fig. 2B). Thus VDR \(^{-/-}\) mice were in a state of sulfate deficiency due to impaired sulfate reabsorption in the kidney.

To examine the effect of chronic sulfate wasting on the organic sulfate pool, the precursor to inorganic sulfate, we measured the levels of hepatic glutathione, a major antioxidant molecule critically involved in cellular detoxification (34). As shown in Fig. 3A, the hepatic glutathione concentration in VDR \(^{-/-}\) mice was moderately but significantly reduced (by 18%) compared with WT mice.

Inorganic sulfate is absolutely necessary for the formation of proteoglycans, the major extracellular matrix component in cartilage. Therefore, we quantified the amount of proteoglycans in the skeleton of VDR \(^{-/-}\) and WT mice. Figure 3B shows that the skeletal sulfated proteoglycan content in VDR \(^{-/-}\) mice was ~45% lower than that in WT mice.

[Graphs and images are not transcribed.]

Fig. 3. VDR \(^{-/-}\) mice have reduced glutathione content in liver and reduced proteoglycan levels in skeleton. A: hepatic glutathione levels in VDR \(^{+/+}\) and VDR \(^{-/-}\) mice. *P < 0.02 vs. VDR \(^{+/+}\) \((n = 5)\). B: proteoglycan levels in skeleton of VDR \(^{+/+}\) and VDR \(^{-/-}\) mice. *P < 0.02 vs. VDR \(^{+/+}\) \((n = 8)\).
Adult VDR<sup>−/−</sup> mice developed hypocalcemia (Fig. 4A) and rickets (Fig. 5, A and B) (25). To determine whether the hypocalcemia contributes to the downregulation of renal NaSi-1 expression and the impairment of sulfate handling, a high-calcium, high-lactose diet was used to normalize the blood ionized calcium levels of VDR<sup>−/−</sup> mice (22). As shown in Fig. 4, 3-mo-old VDR<sup>−/−</sup> mice raised on this special diet had a normal level of blood ionized calcium (Fig. 4A), and their serum parathyroid hormone (PTH) levels were drastically (>90%) reduced (Fig. 4B). The PTH levels were not completely reduced to the levels seen in WT mice because of the lack of VDR-mediated suppression of PTH production (38). In association with the normocalcemia, the rachitic bone phenotype of VDR<sup>−/−</sup> mice was completely prevented by the special diet (cf. Fig. 5, B and D), as shown previously (22). Interestingly, renal NaSi-1 mRNA expression remained lower (by 63%) in the normocalcemic VDR<sup>−/−</sup> mice than in WT mice (Fig. 4C), and intestinal NaSi-1 mRNA levels remained unchanged (data not shown). Moreover, the normocalcemic VDR<sup>−/−</sup> mice still had increased (by 32%) urinary sulfate excretion (Fig. 6A) and reduced (by 48%) serum sulfate levels (Fig. 6B); their skeletal proteoglycan levels remained decreased (by 61%; Fig. 6C), even though the bone structure appeared normal (Fig. 5D). The magnitude of these changes was comparable to that in hypocalcemic VDR<sup>−/−</sup> mice (Figs. 2 and 3).

To confirm the stimulatory role of 1,25(OH)<sub>2</sub>D<sub>3</sub> in NaSi-1 expression, WT mice were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> or a noncalcemic vitamin D analog, RO-27-5646. Injection of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Fig. 7A) or RO-27-5646 (Fig. 7B) caused a robust induction of renal NaSi-1 mRNA. As a control, calcibind-D9k expression in the kidney was also dramatically induced by the treatments (data not shown).

**DISCUSSION**

As one of the most abundant anions in the serum, sulfate is crucial for various physiological functions (27). However, sulfate is far less well studied than other serum ions, and little is known about the factors that regulate sulfate homeostasis in animals. In addition to its principal role in calcium and phosphate homeostasis (9), vitamin D has been shown to regulate a wide range of physiological processes, including immune response (15, 16), hair growth (19, 37), electrolyte, volume, and blood pressure homeostasis (24), as well as mammary gland (43) and muscle development (13). Thus it is not surprising that vitamin D is also involved in sulfate homeostasis. Previous studies show that, in vitamin D-deficient rats, serum sulfate levels were significantly reduced and renal sulfate excretion was markedly increased, accompanied by a reduction in renal NaSi-1 expression (14). However, the physiological impact of vitamin D deficiency...
on the hepatic sulfate pool and skeletal proteoglycan formation has not been reported.

In the present study, we used VDR knockout mice to study sulfate metabolism. We found that, in the genetically mutant mice, renal, but not ileal, NaSi-1 expression is dramatically reduced, which is associated with a significant increase in urinary sulfate excretion and a significant reduction in the levels of serum sulfate, hepatic glutathione, and skeletal sulfated proteoglycans. We further demonstrated that the effect of VDR inactivation on NaSi-1 expression and sulfate metabolism is independent of its effect on calcium metabolism. These results provide solid in vivo evidence supporting a critical role of vitamin D in the regulation of renal sulfate handling. However, the finding that vitamin D regulates NaSi-1 only in the kidney, and not in the intestine, suggests that other cell-specific factors are also involved in this physiologically important regulation. It is also possible that some factors in the intestine play such a predominant role in maintaining NaSi-1 expression that VDR inactivation has no impact on the level of this cotransporter.

The present study demonstrates that VDR inactivation has a negative effect on sulfate status; sulfate wasting as a result of increased sulfate excretion leads to sulfate deficiency. Given the roles that sulfate plays, the physiological impact of sulfate deficiency can be multiple. Here we showed that VDR deficiency causes a dramatic reduction in sulfated proteoglycan synthesis in the skeleton and a moderate decrease in hepatic glutathione levels. The former may represent a direct effect of a decreased inorganic sulfate pool, inasmuch as decreased availability of sulfate may affect intracellular sulfation of cellular components such as proteoglycans. It has been reported that proteoglycan sulfation in articular cartilage is dependent on the inorganic sulfate concentration in the media (42). The latter finding suggests that chronic sulfate wasting may ultimately cause a reduction in the organic sulfate pool, because methionine and cysteine can be metabolized to glutathione, taurine, or inorganic sulfate (21). Glutathione is an antioxidant critically involved in cellular detoxification and reduction-oxidation processes (11, 34), and the consequence of its diminution remains to be determined.

A hallmark of vitamin D deficiency is the development of rickets and osteomalacia. A typical characteristic of rachitic bones is disorganization and expansion of the chondrocyte columns in the growth plate and accumulation of unmineralized bones. These phenotypes are commonly attributed to abnormal calcium and phosphate metabolism caused by impaired vitamin D function (2). Because sulfation is essential for the formation and biological properties of proteoglycans, the major extracellular component of cartilage, it was argued that abnormal sulfate metabolism in vitamin D-deficient animals may contribute to development of rickets and osteomalacia (14). However, our observation that serum sulfate and skeletal proteoglycan levels in normocalcemic VDR−/− mice remained reduced, even in the absence of rickets and osteomalacia, argues against the above notion. That is, the role of sulfate in the development of rickets and osteomalacia is minimal, if any. Certainly, this does not exclude the possibility that the reduction in sulfated proteoglycans may contribute to other, more subtle, bone abnormalities. Further investigations are needed to elucidate the exact role of sulfate in bone growth and remodeling.

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REFERENCES


