Elevated Vitamin D Receptor Levels in Genetic Hypercalciuric Stone-Forming Rats Are Associated With Downregulation of Snail

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ABSTRACT

Patients with idiopathic hypercalciuria (IH) and genetic hypercalciuric stone-forming (GHS) rats, an animal model of IH, are both characterized by normal serum Ca, hypercalciuria, Ca nephrolithiasis, reduced renal Ca reabsorption, and increased bone resorption. Serum 1,25-dihydroxyvitamin D [1,25(OH)2D] levels are elevated or normal in IH and are normal in GHS rats. In GHS rats, vitamin D receptor (VDR) protein levels are elevated in intestinal, kidney, and bone cells, and in IH, peripheral blood monocyte VDR levels are high. The high VDR is thought to amplify the target-tissue actions of normal circulating 1,25(OH)2D levels to increase Ca transport. The aim of this study was to elucidate the molecular mechanisms whereby Snail may contribute to the high VDR levels in GHS rats. In the study, Snail gene expression and protein levels were lower in GHS rat tissues and inversely correlated with VDR gene expression and protein levels in intestine and kidney cells. In human kidney and colon cell lines, ChIP assays revealed endogenous Snail binding close to specific E-box sequences within the human VDR promoter region, whereas only one E-box specifically bound Snail in the rat promoter. Snail binding to rat VDR promoter E-box regions was reduced in GHS compared with normal control intestine and was accompanied by hyperacetylation of histone H3. These results provide evidence that elevated VDR in GHS rats likely occurs because of derepression resulting from reduced Snail binding to the VDR promoter and hyperacetylation of histone H3.

KEY WORDS: GHS RATS; VITAMIN D RECEPTOR; HYPERCALCIURIA; INTESTINE; KIDNEY; SNAIL

Introduction

Idiopathic hypercalciuria (IH) is found in 5% to 7% of the adult population and is the most common risk factor for calcium (Ca) oxalate (Ox) nephrolithiasis. Hypercalciuria is thought to play an important role in the pathogenesis of CaOx stone formation through increased urine supersaturation with respect to Ca and Ox. The disease is characterized by high intestinal Ca absorption and bone resorption and decreased renal Ca reabsorption resulting in hypercalciuria with normal serum Ca, normal or elevated serum 1,25-dihydroxyvitamin D [1,25(OH)2D], normal or low serum phosphate, and low bone mass. Serum parathyroid hormone (PTH) is normal in the vast majority of patients. The familial pattern of IH is consistent with a polygenic mode of inheritance. Candidate genes involved in the pathogenesis of IH have been identified in only a small number of families. The rat homologue of human IH, the genetic hypercalciuric stone-forming (GHS) rat, has a phenotype that exhibits many features with IH, including normal serum Ca, increased intestinal Ca absorption and bone resorption, decreased renal Ca reabsorption, and normal 1,25(OH)2D serum levels, in addition to a polygenic mode of inheritance. Most kidney stones are composed of Ca salts owing to supersaturation of the urine with respect to Ca and the dietary content of anion species such as phosphate and Ox.

All the changes in intestine, kidney, and bone Ca transport in IH may be reproduced by the administration of small doses of 1,25(OH)2D3 to healthy human volunteers. Elevated serum 1,25(OH)2D levels may account for the phenotype in 40% to 60% of IH patients. However, the remaining patients have normal serum 1,25(OH)2D levels and increased Ca transport rates in intestine, kidney, and bone and are indistinguishable from patients with elevated serum 1,25(OH)2D levels. GHS rats share similarities with IH patients with normal serum...
1,25(OH)₂D levels in that elevated intestinal, kidney, and bone VDR protein levels are found in GHS rats, and high VDR levels have been found in the peripheral blood monocytes (PBMs) of male IH stone-formers.

We have postulated that elevated tissue VDR may amplify the biologic actions of normal circulating 1,25(OH)₂D levels and create hypercalcucuria through increased intestinal Ca absorption and bone resorption. 1,25(OH)₂D increases CaR gene expression and may decrease renal tubule Ca reabsorption through activation of CaR. Early generations of GHS rats had elevated duodenal and kidney VDR levels owing in part to prolongation of the half-life of the VDR protein. VDR gene expression by Northern blot analysis was normal or low. In subsequent generations of GHS rats, hypercalcucuria has become more intense, and VDR levels and rates of gene expression are increased in intestine and kidney. Investigations into the causes of elevated VDR in GHS rats have revealed increased transcription rates and prolonged turnover with no difference in the duodenal VDR mRNA sequence.

In this study, the mechanism of upregulation of VDR in GHS rats was explored for evidence of cis- and trans-acting regulation. The transcription factor Snail, a negative regulator of VDR in human colon cancer cells, was investigated for a trans-regulatory role in VDR expression. Snail is a zinc-finger transcription factor expressed in migratory processes during embryonic development and has been implicated in the development of metastatic cancer through downregulation of VDR and the conversion of an epithelial to a mesodermal phenotype. Homozygous Snail gene knockout mice is lethal early in embryogenesis, whereas heterozygous Snail mice appear normal. No information on Snail involvement in mineral homeostasis is available, and Snail regulation of VDR in normal physiology or in the pathophysiology of benign conditions has not been described. To investigate the potential role of Snail in the pathogenesis of the high VDR in GHS rats, we measured Snail gene expression and protein levels in relation to VDR and the binding of Snail to specific sites in the rat VDR promoter regions, as well as the status of histone H3 modification within the proximal VDR promoter region in GHS rats.

Materials and Methods

Animals

The colony of GHS rats was created by the selective breeding of male and female Sprague-Dawley rats (S-D; Harlan, Inc., Indianapolis, IN, USA) with the highest 24-hour urine excretions. Beyond the thirtieth generation, GHS rats have consistently excreted urine containing 10% fetal bovine serum, 50 μg/mL penicillin, 0.25 μg/mL streptomycin, and 2 mM L-glutamine at 37°C under 5% CO₂.

Experimental design

Male GHS and NC rats were fed normal chow containing 1.2% Ca, 0.24% Mg, 1.0% P, and 0.55 μg vitamin D₃ per gram of rat chow. After an overnight fast, rats were placed under deep general anesthesia and exsanguinated via the abdominal aorta. Tissues were harvested, and a portion of each tissue was either rapidly added to RNeasy (Qiagen, Inc., Valencia, CA, USA) for RNA preservation and then stored at −80°C or rinsed in ice-cold PBS buffer, homogenized on ice, and then stored for subsequent Western blot and protein measurements.

Antibodies and vectors

Monoclonal anti-VDR antibody was purchased from Chemicon, Inc. (Temecula, CA, USA). β-actin-specific antibody was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Anti-Snail antibody used for Western blot, electrophoretic mobility gel shift (EMSA), and chromatin immunoprecipitation (ChIP) assays was obtained from Abcam Plc (Cambridge, MA, USA). Secondary antimouse and anti-rabbit horseradish peroxidase (HRP)–linked antibodies were purchased from GE Healthcare UK, Ltd. (Amerham, England). HRP-labeled anti-rat IgG was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Anti-acetylated H₃ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA), and normal IgG was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). pcDNA3 Vector was purchased from Invitrogen Corp. (Carlsbad, CA, USA). pcDNA3-Snail was a generous gift from Dr Amparo Cano (Instituto de Investigaciones Biomédicas UAM/CSIC, Madrid, Spain).

RNA isolation and real-time PCR

Renal and intestinal total RNA were isolated using RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s protocol. Total RNA from cell lines was extracted using Trizol (Invitrogen). First-strand cDNA synthesis was performed with an oligo dT primer using a cDNA synthesis kit (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA, USA). Gene-specific oligonucleotides used for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) were designed using Primer Premier V. Blast searches were used to ensure that primers were specific for each individual gene. Real-time PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7700 apparatus (Perkin Elmer, Applied Biosystems). Thermocycling was done in a final volume of 25 μL containing 60 ng of cDNA sample, 800 nM of each of the forward and reverse primers. The PCR was performed as the following program: 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 65°C for 20 seconds, and 72°C for 30 seconds. The relative amount of each sample was calculated from a standard curve after employing the fit-point algorithm for quantification. A relative standard curve was generated from pooled RNA of NC and GHS samples. For accurate relative
quantitation, the dilution series from which the standard curve was generated was carefully prepared using dilution factors of 1-, 3-, 9-, 27-, and 81-fold. The expression value was normalized to GAPDH or β-actin. A relative gene expression was determined by assigning the adult rat groups fed a normal calcium diet (NCD) a relative value of 1.0, with unknown values relative to this control. Primer sequences are listed in Table 1.

DNA preparation and sequencing reaction

Genomic DNA was purified using the Puregene DNA purification kit (Genta Systems, Inc., Minneapolis, MN, USA). For SNP discovery, PCR was used to amplify the approximate 7.3 kb fragment containing the promoter, all exons, and the exon/intron boundary regions. Primers were designed according to the reference sequence AF288738 from GenBank using Primer Premier Version 5.00 (Premier Biosoft International, Palo Alto, CA, USA) (Table 2). PCR was carried out using 1 unit of Hotstar Taq polymerase (Qiagen, Inc.) with 4 mM Mg²⁺, 20% Q-solution, and 20 ng DNA in 40 μL final volume. A touchdown thermal cycling protocol was used for all amplification: 98°C for 10 minutes for denaturation and activation of DNA polymerase followed by 8 cycles of denaturation at 98°C for 20 seconds, annealing at 68°C for 20 seconds and 1°C decreasing per cycle, and extension at 72°C for 40 seconds. After touchdown, another 35 cycles were performed at 98°C for 20 seconds, 59°C for 20 seconds, and 72°C for 40 seconds. PCR products were purified with Qiaquick PCR purification Kit (Qiagen, Inc.), followed by direct sequencing from both ends using Bigdye Terminator Kit Version 3.0 (Applied Biosystems). Sequencing reaction products were precipitated and then separated on ABI-3700 capillary sequencer (Applied Biosystems). The GHS VDR sequences were compared with the NC VDR sequences using blast 2 (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Protein extraction and Western blot

Tissue was homogenized in cold lysis 250 buffer (50 mM Tris/HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM PMSF, 1 μg/mL leupeptin, and 1 μg/mL antipain) and then chilled on ice for 30 minutes. The mix was vortexed at full-speed for 20 seconds every 5 minutes and then centrifuged for 15 minutes at 14,000 × g at 4°C. The supernatant was removed and stored at −80°C. Nuclear extracts were isolated from cell lines using the NE-PER kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total protein aliquots of 20 μg were denatured in 6× SDS sample buffer (7 mL 4× Tris/HCl, SDS, pH 6.8, 3.0 mL of glycerol, 1 g of SDS, 0.93 g of dithiothreitol, and 1.2 mg of bromphenol blue in 10 mL of distilled deionized H₂O) and then loaded onto SDS denaturing discontinuous gels. The protein was transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA, USA) by electroblotting at 90 V for 1 hour. The membranes then were blocked with 5% nonfat dried milk in TBS that contained 0.1% Tween-20 (TBS-T).

Table 2. Primers Used for Sequencing GHS and NC Rat VDR Exons, Intron/Exon Boundary Regions, and Proximal Promoter

| Primer | Forward Primer | Reverse Primer |
|--------|----------------|---------------|
| 1      | CCAATCGTTCCCTTTCTTACTCC | GGTCTCCAAAGGCGACAGTGCA |
| 2      | CAAACACGCCAACATCTGCTTG | AAAGACACGACCTTACCAGAAAA |
| 3      | AGACGGGAATGGAATGGTGG | TTTGTTCATTGAGGACAGTCTC |
| 4      | GAGTTGGAGAATCTGAGTGTAGTTG | AATCAGTTGGATATGTGTTGCT |
| 5      | CAGGAGGAAATCTCAACAGC | TCCAGAAGATTTCCTGCCACCT |
| 6      | CCGAGGAAAAAGAACAAAGCAG | CAAGGCGGACAGTGGGCAGT |
| 7      | AATCATCCTTGTGACATCAAC | CGAGCTCGGAGGCTTCTCTA |
| 8      | ATGCCCTTCTTCTGCTTGTA | GCTGCCCCTCTCGCTTGA |
| 9      | GCCCTGTTGGCTGCTGTA | CCAAAAAAAGTTCACCCACT |
| 10     | ACCTCGTCATTATTAGGGCCTCTCT | TGGTGCTGCTGCTGCTCACA |
| 11     | CCTCTTCTTTAGCCTGAGAC | GAAACCCAGTGTCTTCCATG |
| 12     | GGTTCAGTCAGGCGACTTCT | GGAAGAGGCTACTTATGG |
| 13     | TGCAAAACCCAGAAAAGTGAA | GGTTGGACCTCTGCCCAAA |
| 14     | AGAGTGGGCAAGACAAGAGACAG | GAAGAGAAGGTGAGGAGTG |
| 15     | TTTCCAATGGTGTTTCTAGG | CACAGTGTTGCTGGAGT |

Table 1. Primers Used for Real-Time PCR

| Primer       | Forward Primer | Reverse Primer |
|--------------|----------------|---------------|
| Rat VDR      | GCCCTCTAAAGGTCCAGGTG | GGTAGGCGGTCTCTGAAAGTG |
| Rat Snail    | CTGGGGCGCCCTGGAAGATC | GGAAGCCGAGCCTTTGCTG |
| Rat GAPDH    | GCCAGCTCTGCTCTAGACA | AGAGAAGGCGGCTCTGGTAAC |
| Human VDR    | TCCCTCTGCTGACATCTG | AGGTTCAGAAGGGCTG |
| Human Snail  | CCAACTGGGAAGCTGAACTA | GCGCTGCTGGAGGTAAACTC |
| Human β-actin| TGGACTTCGGAGAAGATG | GAAGGAGGCTGGAAGAGTG |
The primary antibody was diluted with 3% nonfat dried milk in TBS-T. Then the membrane was incubated with the diluted primary antibody at room temperature for 1 hour. Membranes were washed with TBS-T and incubated with HRP-conjugated anti-rat IgG for 1 hour at room temperature. Band intensities were developed using the ECL Chemiluminescence System (Amersham Life Science, Buckinghamshire, UK). The blots were quantified by scanning using One-Scan 1D Gel Analysis software (Scangalitics, Inc., Fairfax, VA, USA).

**EMSA assay**

Human proximal tubule–derived HEK293 cells were transfected with pcDNA3 and pcDNA-Snail using Lipofectamine and Lipofectamine 2000 Transfection Reagent (Invitrogen). After 48 hours of transfection, nuclear extracts were isolated using the NE-PER (Pierce Biotechnology), and Snail protein was determined by Western blot.

In the EMSA assay, the double-strand oligonucleotides probes were labeled with biotin. Nonlabeled oligonucleotides were used as competitors for biotin-labeled oligonucleotide binding. The sequences flanking the E-boxes in the rat VDR promoter region are for E-box 1, 5'-GGTCAACTCAGGTACGGGTGACACAC-3'; for E-box 2, 5'-ACGGGGTCACACCGTCCGAGGCGGT-3'; and for E-box 3, 5'-GGGGCCGGGCGGAGGGCAGTC-3'. Briefly, 20 μL of binding reaction containing 2 μg of nuclear extract was prepared according to the kit instruction manual and incubated at room temperature for 20 minutes. Complexes were resolved on 4% acrylamide gels (29:1 acrylamide:bisacrylamide). When electrophoresis was completed, binding reactions were electrophoretically transferred to a nylon membrane. The transferred DNA was then cross-linked to the nylon membrane, and the biotin-labeled DNA-protein complex was detected by chemiluminescence kit (Pierce Biotechnology). For antibody inhibition of protein-DNA interaction, anti-Snail antibody was added to the binding reactions prior to electrophoresis and incubated for 20 minutes at room temperature. The reaction mix then was resolved and transferred as described earlier.

**ChIP Assay**

ChIP assays were performed on DNA isolated from HEK293, SW480, and DLD1 cell lines and from frozen intestine and kidney tissues as described previously with some modifications. Using the cell lines, cells were cross-linked by adding 270 μL formaldehyde into 10 mL of medium for 10 minutes and then stopped by addition of glycine at a final concentration of 0.125 M. Cell lysates then were sonicated into 200 to 1000 bp DNA fragments. Anti-Snail antibody was used to immunoprecipitate the DNA-protein complex, and IgG alone was used as a negative control. After overnight incubation with antibody, immunocomplexes were collected using 80 μL of protein A agarose beads in a slurry (Upstate Biotechnology, Lake Placid, NY, USA). The agarose beads were washed twice with the following buffers (Upstate Biotechnology): low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and TE buffer. DNA was eluted with 1% SDS and 0.1 M NaHCO₃ elution buffer, subjected to reverse cross-linking, proteinase digestion, and purification using a commercial kit (Qiagen, Inc.). Primers used to amplify the human VDR promoter region are 5'-CTGCCAGCTAAACAGTTTG-3' and 5'-GCTTGAGCTGTGGTATCG-3'.

For the tissue ChIP assay, frozen kidney and intestine tissues from GHS and NC rats were thawed at room temperature, cut into 1 mm slices, and crossed-linked in 1.5% formaldehyde for 15 minutes. Tissues were then homogenized with TissueMixer (Fisher Scientific, Pittsburgh, PA, USA), and the cell suspension was centrifuged at 14,000 rpm for 10 minutes. SDS lysis buffer was added to the pellets, and they were processed as described earlier except that antibodies against acetylated H3 were used. The primers that amplify the rat VDR promoter region are 5'-CCACCCCATAGTTCCAGGTCT-3' and 5'-CGGACTCCACTGGTAGGAT-3'.

**Immunostaining assay**

Small intestine and kidney tissues of GHS rats were harvested, embedded in Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA, USA), and snap frozen with liquid nitrogen. Then 5 μm sections were cut with a cryostat. An indirect double-fluorescence method was applied to detect the expression patterns of VDR and Snail in intestinal mucosal epithelial crypt and villus cells and in kidney segments from the loop of Henle and distal convoluted tubule regions of the nephron. In brief, intestinal and kidney slices were fixed with 4% paraformaldehyde and blocked with 5% bovine serum albumin (BSA) to eliminate nonspecific staining. For intestinal crypt and villus staining, sections were sequentially incubated with mixed rat anti-VDR monoclonal antibody (1:100; Millipore Corp., Bedford, MA, USA) plus rabbit anti-Snail polyclonal (1:100; Abcam Plc, Cambridge, MA), biotinylated mouse anti-rat IgG2b (4 μg/mL; BD Pharmingen, San Diego, CA, USA), fluorescein isothiocyanate (FITC)–conjugated streptavidin (1:100; BD Pharmingen), and then Texas red conjugated goat anti-rabbit IgG (1:100; Jackson Immunoresearch Labs, West Grove, PA, USA). For staining of loop of Henle and distal convoluted tubule sections, rabbit anti-human VDR polyclonal antibody was obtained from Abcam Plc and used at a dilution of 1:200. Goat anti-human Snail polyclonal antibody was obtained from Santa Cruz Biotechnology and applied at a dilution of 1:100 in PBS as primary antibody and incubated overnight. Texas Red conjugated goat anti-rabbit IgG (1:100, Jackson Immunoresearch Labs) and FITC-conjugated rabbit anti-goat antibodies (1:100, BD Pharmingen) were applied and incubated for 30 minutes at room temperature.

**Data analysis**

In our study, the Spearman’s rank correlation between Snail and VDR was computed, and its statistical significance was assessed. A nonparametric approach was used in this assay. Gene expression data distribution was normalized by transformation by log₁₀ for statistical analysis. In all statistical tests, two-tailed p values of .05 or less were considered statistically significant.

**Results**

VDR protein and gene expression levels in GHS rat intestine and kidney

Semi-quantitative immunoblotting demonstrates a significant increase in VDR protein levels in GHS rat duodenum and kidney
VDR protein levels were elevated 9.9-fold in jejunum and 6.2-fold in ileum from GHS compared with NC rats (Fig. 1A). In GHS rats, elevated VDR protein levels were comparable across duodenum, jejunum, ileum, and kidney cortex.

Using primers for VDR (see Table 1), real-time PCR confirmed the previously reported increase in VDR mRNA in GHS rat kidney cortex (see Fig. 1B). VDR mRNA was statistically significantly increased in GHS rat duodenum and jejunal but not ileal mucosa (see Fig. 1B). VDR mRNA expression levels varied by intestinal segment and were lower in kidney compared with all three regions of the small intestine analyzed. Duodenal VDR mRNA levels were increased 3.1-fold in GHS rats, whereas VDR protein levels were increased 6.3-fold. In GHS rat kidney, VDR mRNA and protein levels were raised 2.5- and 5.5-fold, respectively. The greater magnitude increase in VDR protein compared with mRNA in GHS rat duodenum and kidney supports previous observations that increased VDR levels may be due to factors in addition to transcriptional regulation, including altered efficiency of VDR translational events and the stability of the VDR protein. Indeed, prolonged half-life of the GHS rat duodenal and renal cortical VDR protein was described in a previous study conducted in vivo.

Sequencing the GHS rat VDR exons, intron/exon boundary regions, and proximal promoter region

Samples of kidney genomic DNA from four GHS and two NC rats were subjected to DNA sequencing (data not shown). The results revealed no mutation, polymorphism, or splicing variant in the 5' UTR exons, CDS exons, intron/exon boundary regions, or 3' UTR exons of the VDR. There was no difference in the DNA sequence of the proximal 2 kb of the VDR proximal promoter region between the two groups. Therefore, the high mRNA levels of VDR in GHS rats may not result from mutation or allelic variation in the cDNA or proximal promoter regions. However, the studies do not exclude the possible regulation of VDR expression by an enhancer located far upstream, downstream, or within an intron.

Snail expression is downregulated in GHS rats

To assess potential trans-regulation of VDR expression in GHS rats, mRNA analysis of Snail was performed. Immunoreactive Snail protein was faintly detectable by Western blot using whole-cell extract (data not shown) and was readily detectable in nuclear extracts from GHS and NC rats (Fig. 2A), with a marked decrease in protein in GHS rat duodenal mucosa. Snail protein levels were decreased in (A) duodenum and (B) kidney. (C) Total RNA isolated from intestinal and kidney tissue from GHS and NC rats was subjected to real-time PCR using primers listed in Table 1. Snail mRNA levels in GHS rat duodenum (p = .026) and kidney (p = .002) were decreased (n = 4). Each data point represents intestinal or kidney extracts from a single rat. Snail levels in jejunum and ileum were not statistically significantly different between GHS and NC rats.

Fig. 1. VDR is highly expressed in GHS rat intestine and kidney at the protein and mRNA levels. (A) Immunoblot for VDR and β-actin in total nuclear protein extracted from intestinal and kidney tissue from GHS and NC rats. Each lane represents intestinal or kidney extracts from an individual rat. All samples were run on the same blot. Lanes 1 to 4 are from NC rats; lanes 5 to 8 are from GHS rats. Lanes 1 and 5 are duodenum; lanes 2 and 6 are jejunum; lanes 3 and 7 are ileum, and lanes 4 and 8 are kidney. (B) Intestine and kidney VDR and GAPDH mRNAs from GHS and NC rats were subjected to real-time PCR using the primers in Table 1. VDR expression levels were normalized to GAPDH expression. VDR mRNA levels are increased in GHS versus NC rat duodenum, jejunum, and kidney. Results are individual values with mean shown as horizontal bars (n = 4 per group). p = .027; ***p = .034; **p = .036.

Fig. 2. Snail is decreased in GHS rats at the protein and mRNA levels. (A, B) Immunoblots for Snail and β-actin of nuclear protein extracts from duodenum and kidney from individual GHS and NC rats. Representative results from an individual GHS and NC rat are shown. Snail protein levels were decreased in (A) duodenum and (B) kidney. (C) Total RNA isolated from intestinal and kidney tissue from GHS and NC rats was subjected to real-time PCR using primers listed in Table 1. Snail mRNA levels in GHS rat duodenum (p = .026) and kidney (p = .002) were decreased (n = 4). Each data point represents intestinal or kidney extracts from a single rat. Snail levels in jejunum and ileum were not statistically significantly different between GHS and NC rats.
was faintly detectable in nuclear extracts of renal cortical tissue from NC but not GHS rats. In GHS rats, Snail mRNA levels were suppressed 7.5-fold in duodenum, 2.2-fold in jejunum, 2.0-fold in ileum, and 6.7-fold in kidney, with only the duodenal and kidney suppressions being statistically significant (see Fig. 2B). When measurements of Snail and VDR mRNA levels from the same tissue obtained from individual GHS and control rats were compared, there was a significant inverse relationship between Snail and VDR expression (Fig. 3). Snail and VDR expressions were inversely correlated by the Spearman rank correlation coefficient, where $r = -0.507$ and $p = .0031$.

Snail binds to the E-box 3 within the rat VDR proximal promoter region

Wild-type mouse Snail was cloned into mammalian vector pcDNA3 (pcDNA3-Snail) and then transiently transfected into HEK293 cells. Nuclear extracts from the HEK293 cells transfected with pcDNA3-Snail contained a greater increase in Snail protein after 48 hours than cells that were transfected with the pcDNA3 empty vector (Fig. 4A). EMSA and antibody-inhibition assays were performed using nuclear extracts from the transfected HEK293 cells. In the EMSA assay, the unlabeled rat E-box 1 (see Fig. 4B) and rat E-box 2 (see Fig. 4C) probes did not compete with the labeled probes for binding to the DNA-protein complexes. These results indicate that Snail does not bind specifically to the rat E-box 1 and 2 sequences. However, a specific DNA-protein binding complex was demonstrated when the rat E-box 3 DNA probe was incubated with pcDNA-Snail-transfected nuclear extracts (see Fig. 4D). When excess nonlabeled E-box 3 oligonucleotides were added to the incubation, the DNA-protein binding complexes were reduced. Furthermore, anti-Snail antibody inhibited the Snail DNA-protein binding, whereas the control using normal IgG was without effect (see Fig. 4D). These results indicate that Snail specifically binds to rat E-box 3.
The EMSA data suggest that Snail could regulate VDR transcription through binding to the E-box 3 site located within the VDR promoter.

Snail binds to the VDR promoter in vivo

Snail and VDR mRNA and protein levels in HEK293, DLD1, and SW480 cells were measured by real-time PCR (Fig. 5A) and Western blot (Fig. 5B). VDR mRNA levels were significantly higher and Snail mRNA levels significantly lower in DLD1 and SW480 cells compared with HEK293 cells (see Fig. 5A). VDR protein levels also were higher in DLD1 and SW480 cells than in HEK293 cells (see Fig. 5B), and Snail protein levels were lower in DLD1 cells than in HEK293 and SW480 cells (see Fig. 5B). The statistically significant differences in Snail and VDR mRNA levels across the three cell lines with higher VDR levels associated with lower Snail levels strongly suggest that the inverse relationship between Snail and VDR levels found in the tissues from GHS and NC rats (Figs. 1 through 3) also may exist within the cell lines examined. Based on the evidence that Snail binds to the human VDR promoter in vivo, we used the three human cell lines (noted earlier) that varied in VDR content to explore Snail binding to the VDR promoter. Endogenous Snail occupied the VDR promoter in SW480 and HEK293 cells, but no Snail binding was detected in DLD1 cells (see Fig. 5C), in which Snail expression was very low (see Fig. 5A). The primers used to amplify the VDR promoter sequence included the E-boxes. Therefore, our results strongly suggest that Snail regulates VDR expression through binding to the VDR promoter in vivo.

Hyperacetylation of histone H3 around GHS rat VDR promoter

Snail gene expression and protein levels are downregulated in GHS rat intestine and kidney (see Fig. 2). Tissue ChIP assays revealed Snail binding to the VDR promoter region close to E-boxes in intestine of NC rats, with weaker binding in GHS rat intestine (Fig. 6A). Hyperacetylation of histone H3 was observed in the same VDR promoter region in intestine and kidney of GHS rats (Fig. 6B).

Coexpression of VDR and Snail in small intestinal epithelial cells of GHS rats

VDR is expressed in intestinal villus cells (Fig. 7A, C) and crypt cells (Fig. 7D, F) and localizes within the epithelial nuclei. Snail has the same expression pattern within the intestinal epithelia and colocalizes with VDR in the same nuclei (see Fig. 7B, C, E, F).
Localization of VDR and Snail in the loop of Henle and distal convoluted tubules in GHS rats

Fluorescence staining of VDR and Snail revealed that both were expressed in the epithelial cells composing the loop of Henle (Fig. 8A–C) and distal convoluted tubule (Fig. 8D–F). Furthermore, VDR (see Fig. 8A, D) and Snail (see Fig. 8B, E) were coexpressed (see Fig. 8C, F) in the same renal epithelial cells in both nephron segments. VDR was expressed in a greater epithelial population and with higher signal intensity than Snail.

Discussion

In GHS rats, hypercalciuria results from simultaneous increases in intestinal Ca absorption and decreased tubule Ca reabsorption. Under physiologic and pharmacologic conditions, 1,25(OH)2D3 increases intestinal Ca transport, increases urine Ca excretion, and stimulates osteoclast-mediated bone resorption. However, serum 1,25(OH)2D levels are normal and not elevated in GHS rats, and therefore, excess circulating 1,25(OH)2D cannot account for the increases in Ca transport that results in hypercalciuria. An alternative hypothesis that elevated tissue VDR levels may amplify the biologic effects of normal serum 1,25(OH)2D levels was confirmed in GHS rats by demonstrating elevated VDR levels in intestine, bone, kidney, and splenic monocytes (personal communication). Elevated intestinal calbindin 9 kDa renal calbindin 28 kDa and renal Ca-sensing receptor gene expressions and protein levels support the hypothesis that elevated tissue VDR levels are functional and serve to amplify the biologic actions of normal circulating 1,25(OH)2D levels through increased expression of vitamin D-dependent genes whose protein products regulate cellular Ca transport.

This study explores potential mechanisms that may elevate VDR levels in GHS rat tissues. In previous studies in GHS rats, the in vivo half-life of VDR protein and VDR mRNA in duodenum and kidney were observed to be prolonged, and this may be responsible for at least a portion of the elevated VDR protein and mRNA levels. In this study, semiquantitative real-time PCR detected several-fold increases in VDR mRNA levels in GHS rat duodenal and renal cortical tissue (see Fig. 1B). Regional increases in intestinal VDR mRNA levels also were noted, with elevated levels in duodenum and jejunum. Thus elevated tissue VDR levels can result from both increased VDR gene expression and protein synthesis and prolongation of VDR protein and mRNA half-life. The relative contributions of extended VDR half-life and increased VDR gene expression to total VDR protein tissue levels have not been studied specifically. However, it is worth noting that in this study GHS rat duodenal VDR gene expression was increased 3.1-fold compared with control (NC).
rats, whereas VDR protein levels were increased 6.3-fold. The level of duodenal VDR protein in this study was greater than reported in studies using earlier generations of GHS rats (two- to fourfold increase), suggesting that the progressive increase in tissue VDR content with time may be due in part to increased VDR gene expression.

The mechanism whereby VDR gene expression is increased in GHS rats was further investigated by VDR gene DNA sequencing. Previous reports of normal cDNA sequence of the VDR mRNA and normal migration of the VDR protein by Western blot suggested that a mutation or allelic variation of the VDR gene likely was not responsible for VDR gene overexpression in GHS rats. In this study, DNA sequencing of the intron/exon boundaries and the 5’ flanking promoter region of the VDR gene in GHS rats failed to demonstrate any DNA sequence differences between the GHS rats and wild-type controls. Further, no allelic variation or mutation was identified that could account for the observed upregulation in VDR gene expression. A recent study indicates that 1,25(OH)2D3 autoregulates mouse VDR genes expression through binding to three enhancer sites located 20 to 29 kb downstream of the transcriptional start site and within the VDR gene. Since we did not sequence the introns of the VDR gene in this study, we have not excluded the possibility of an enhancer-sequence variation in one or more introns that may increase the transcription of VDR in GHS rats.

In the absence of evidence for a mutation or allelic variation of VDR in GHS rats, we considered a dysregulation of a normal VDR gene. To this end, a recent study of human colon cancer tissues in which repression of VDR gene expression was accompanied by overexpression of the Snail gene raised the possibility of an inverse relationship between VDR and Snail gene expression in GHS rats. Snail is a zinc-finger transcription factor that is expressed in migratory processes during embryonic development and has been implicated recently in the phenotypic changes in colon cancer cells from an epithelial to a mesodermal morphology as the cells take on metastatic potential. In the colon cancer cells, upregulated Snail is inversely correlated with cellular dedifferentiation and low VDR expression. In this study, GHS rat tissues we examined had high VDR gene expression that was accompanied by repression of Snail gene expression. Thus the increased VDR tissue levels appear to be due to elevated gene expression of a normal VDR gene, and the elevated VDR gene expression is inversely related to suppression of the Snail gene. The mechanism whereby suppression of Snail could enhance VDR expression centered around the interaction of Snail and E-box binding sites within the VDR promoter.

The rat VDR promoter is highly analogous to the human VDR promoter (Table 3), with particular high homology of the DNA sequences of the E-boxes and their flanking regions. The consensus binding site for Snail-related genes contains a core of six bases (CAGGTG) with a sequence motif that is identical to the E-box, which is the consensus core-binding site of the basic helix-loop-helix (bHLH) transcription factors. The sequence indicates that Snail protein might compete with the bHLH factors.
and 2. The specific binding pattern in the GHS rat binding of Snail protein at the rat E-box 3 but not at E-boxes 1 within the GHS rat binding site for transcriptional repression by Snail of 2 (see Table 3). The data suggest that the E-box 3 is a putative may be due to conserved DNA sequence of E-box 3 but not 1 and regulated target genes for transcriptional repression that under the pathologic condition in GHS rats, lower levels of acetylation of histone H3 around the rat VDR promoter is associated with lower VDR expression. Furthermore, greater expression of Snail removes tonic repression of its overexpression. The cause of reduced VDR expression lessens its suppressive actions and may result in VDR upregulation. To our knowledge, this is the first example of dysregulation of the Snail gene in a noncancerous genetic disorder.

Disclosures

All the authors state that they have no conflicts of interest.

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