Importance of apical membrane delivery of 1,25-dihydroxyvitamin D₃ to vitamin D-responsive gene expression in the colon

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Koszewski NJ, Horst RL, Goff JP. Importance of apical membrane delivery of 1,25-dihydroxyvitamin D₃ to vitamin D-responsive gene expression in the colon. Am J Physiol Gastrointest Liver Physiol 303: G870–G878, 2012.—Synthetic conjugation of a glucuronide to 1,25-dihydroxyvitamin D₃ (1,25D₃) to produce β-25-monoglucuronide-1,25D₃ (βGluc-1,25D₃) renders the hormone bio-logically inactive and resistant to mammalian digestive enzymes. However, β-glucuronidase produced by bacteria in the lower intestinal tract can cleave off the glucuronide, releasing the active hormone. In mice given a single oral dose of 1,25D₃, 24-hydroxylase (Cyp24a1) gene expression was strongly enhanced in the duodenum, but not in the colon, despite circulating concentrations of 1,25D₃ that peaked at ~3.0 nmol/l. In contrast, in mice treated with an equimolar dose of βGluc-1,25D₃, Cyp24a1 gene expression increased 700-fold in the colon but was significantly weaker in the duodenum compared with mice treated with 1,25D₃. Similar results were observed with another vitamin D-dependent gene. When administered subcutaneously, 1,25D₃ weakly stimulated colon Cyp24a1 gene expression while βGluc-1,25D₃ again resulted in strong enhancement. Surgical ligation to block passage of ingesta beyond the upper intestinal tract abolished upregulation of colon Cyp24a1 gene expression by orally and subcutaneously administered βGluc-1,25D₃. Feeding βGluc-1,25D₃ for 5 days revealed a linear, dose-dependent increase in colon Cyp24a1 gene expression but did not significantly increase plasma 1,25D₃ or calcium concentrations. This study indicates that the colon is relatively insensitive to circulating concentrations of 1,25D₃ and that the strongest gene enhancement occurs when the hormone reaches the colon via the lumen of the intestinal tract. These findings have broad implications for the use of vitamin D compounds in colon disorders and set the stage for future therapeutic studies utilizing βGluc-1,25D₃ in their treatment.

IN ADDITION TO ITS WELL-KNOWN endocrine actions on calcium metabolism, 1,25-dihydroxyvitamin D₃ (1,25D₃) can regulate cell proliferation and differentiation (3, 9, 47). Epidemiological evidence suggests that the prevalence of widespread vitamin D insufficiency may be linked to the development of several different types of cancer (15, 51), particularly colon cancer (12, 16, 18, 26, 31, 37, 40). Human colon cancer cell lines, such as HT-29 (55) and Caco-2 (5), and the mouse colon cancer cell line MC-26 (34) respond to the antiproliferative and pro-differentiating effects of 1,25D₃ with reduced in vitro growth. Similarly, inflammatory bowel disease (IBD) has been shown to be linked to poor vitamin D status, and mouse models of IBD respond to 1,25D₃ treatment (4, 20, 33, 43). Unfortunately, the doses of 1,25D₃ required to effectively treat tumors or IBD in vivo result in strong stimulation of the vitamin’s classical effects on calcium homeostasis and the development of life-threatening hypercalcemia (23, 45).

Since many cancerous cell lines respond to 1,25D₃ treatment in vitro, a great effort is being made to develop “nonhypercalcemic” analogs of 1,25D₃ to treat tumors (32, 41). Mice carrying xenografts of various cancer cell lines have been treated with some of these new analogs and have exhibited improved suppression of tumor growth, but even these drugs eventually caused hypercalcemia at doses that proved most effective at causing tumor regression (30, 46, 53). A phase I trial of the nonhypercalcemic analog EB1089 given to human colon cancer patients in the final phase of life (US Food and Drug Administration compassionate treatment lasting a few months) demonstrated some stabilization of tumor growth, but the study also indicated dose-dependent development of hypercalcemia (22). So, despite these promising results, there is still a need to find or develop compounds that can target vitamin D activity to the colon while avoiding hypercalcemia.

Glucuronide conjugates of vitamin D were recognized in bile secretions years ago and suggested a role for enterohepatic circulation in the metabolism of vitamin D (17, 36, 52). However, their importance in circulating vitamin D status and calcium homeostasis was generally dismissed (8). Nevertheless, on the basis of the earlier studies and our prior experience with glycoside derivatives of 1,25D₃ (6), we synthesized a prodrug version of 1,25D₃ by conjugating a glucuronide molecule to a β-linkage to form β-25-monoglucuronide-1,25D₃ (βGluc-1,25D₃). The glucuronide β-linkage limits binding of βGluc-1,25D₃ to the vitamin D receptor (VDR) to ~1% of that observed with the native hormone; therefore, βGluc-1,25D₃ is biologically inactive (44). It is also too large and too watersoluble to be absorbed across the small intestine (39), and mammalian digestive enzymes of the upper intestinal tract are incapable of cleaving the glucuronide from 1,25D₃ (20). However, once the prodrug reaches the lower intestinal tract, β-glucuronidase enzymes produced by bacteria residing in the lumen of the ileum and colon can cleave the glucuronide from the conjugate and liberate 1,25D₃ to act on cells of the lower intestinal tract. Work from our laboratory demonstrated that orally administered βGluc-1,25D₃ ameliorated the severity of colitis in mice with experimentally induced IBD (20). We found that the glucuronide compound also did not significantly raise blood concentrations of 1,25D₃ and calcium (20). Furthermore, βGluc-1,25D₃ appeared to act selectively to enhance vitamin D-dependent gene expression in the colon as opposed to the duodenum. In contrast, 1,25D₃ treatment strongly stimulated target gene expression in the duodenum; however, it was unexpectedly much less effective at inducing expression in the colon, despite high circulating concentrations of the hormone. The present study sought to examine these initial findings in more detail and document the activity and selectivity of βGluc-
1,25D$_3$ vs. 1,25D$_2$ in the intestinal tract as a prelude for the possible use of βGluc-1,25D$_3$ in the treatment of colon cancer and IBD.

**MATERIALS AND METHODS**

**Vitamin D compounds.** βGluc-1,25D$_3$ (mol wt 592.76) was synthesized as described previously (20). It was purified by high-performance liquid chromatography to >97% purity, and the structure was confirmed by Fourier transform infrared spectroscopy and NMR. The 1,25D$_3$ (mol wt 416.64) was purchased from Sigma Aldrich (St. Louis, MO) and was >98% pure. Quantitation of vitamin D compounds in ethanol solutions utilized to prepare each treatment was based on absorbance at 264 nm using a molar extinction coefficient of 18,200 absorbance units-mol$^{-1}$-cm$^{-1}$.

**Tissue culture.** LNCaP cells (CRL-1740, American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium containing 10% fetal bovine serum with penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C. Cells were seeded into a six-well plate and treated with vehicle, 100 nM 1,25D$_3$, or 100 nM βGluc-1,25D$_3$ for 48 h. Medium was removed, and the cells were disrupted by direct treatment with 1.0 ml of TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was processed and reverse-transcribed as described below, and PCR analysis of human 24-hydroxylase (Cyp24a1) and Gapdh genes was performed using the following primers: humCyp24 [5'-CAGGTGCCACGGCGCAAGA (forward) and 5'-CCTGGAT-GTGGTATTGCGGACAA (reverse)] and humGapdh [5'-CAGCCTC-CCTGGATGAC-86°C prior to processing for RNA.

**RNA processing.** Each TRIzol homogenate was thawed at room temperature, and 500 µl were placed in a clean microfuge tube, mixed thoroughly with 100 µl of chloroform for 15 s, and then centrifuged at 12,000 g for 15 min at 4°C. The upper aqueous phase was removed and mixed with 0.93 volume of 75% ethanol. The mixture was applied to an RNasey spin column (Qiagen, Germantown, MD) and processed as described by the manufacturer, with an additional wash with 2 M NaCl-2 mM EDTA (pH 4.0) (11). RNA was eluted in 50 µl of water, and the concentration was obtained by UV spectrometry. Then 1 µg of RNA was used as a template for production of cDNA in a 20-µl reaction volume using random hexamers and SuperScript III (Invitrogen) or QuantiTect PCR (Qiagen, Valencia, CA), as described by the manufacturers. The samples were diluted to a final volume of 100 µl with Tris-EDTA buffer and stored at −20°C prior to PCR analysis.

**Quantitative real-time PCR.** Quantitative real-time PCR (qRT-PCR) analysis was performed using a thermal cycler (model MX3005p, Stratagene, La Jolla, CA) and PerfeCTa SYBR Green FastMix, Low ROX reagent (Quanta Biosciences, Gaithersburg, MD). Amplification of murine target cDNAs was accomplished with the following primers (synthesized by Integrated DNA Technologies, Coralville, IA): mouse Cyp24 [5'-CACACGCTGCGCTGGGACAC (forward) and 5'-GGGACTCGTGACAGAGCG (reverse)], mouse Gapdh [5'-GAAGGTCGTTGAGCCAGGATTGGGC (forward) and 5'-TTGATGTATGTTGAGGTCAGTCGCTCTG (reverse)], and mouse transient receptor potential cation channel subfamily V member 6 (Trpv6) [5'-GCGGACAGCAAGACAGCTG (forward) and 5'-GAGCTTGCTCAGGCTCAGCG (reverse)]. Similarly, amplification of rat target cDNAs was accomplished with the following primers: rat Cyp24 [5'-AACACGCTGCGCTGGGACAC (forward) and 5'-TGTTGCTGCTGTGCTTGACT (reverse)] and rat Gapdh [5'-CCTGGACACAACTTGTCGCTG (forward) and 5'-GCCAGTG-GAGCTCGTGACAGAGCG (reverse)]. Aliquots (8.3 ng) of cDNA were amplified under the following conditions: 95°C for 30 s followed by 45 cycles of 95°C for 1 s and 57°C for 30 s. All reactions were performed in duplicate, with three to five animals per treatment, and target gene expression was estimated using the cycle threshold (ΔCt) method normalized to Gapdh expression, as described previously (19, 20).

**Plasma 1,25D$_3$ analysis.** Mice and rats were euthanized by guillotine while under isoflurane anesthesia. Blood was collected from the
cervical stump into heparinized tubes, and plasma was collected and frozen at −86°C until analysis. Because βGluc-1,25D₃ is more water-soluble than 1,25D₃ and, therefore, elutes with the methanol wash of the 0.5-g C₁₈OH solid-phase extraction column (Varian, Lexington, MA), it is possible to measure only 1,25D₃ in the samples. Plasma 1,25D₃ concentrations were determined by radioimmunoassay on individual samples of plasma collected from the mice and rats (Heartland Assays) (27, 28). Calcium content was determined by colorimetric assay (Arsenazo III, Pointe Scientific, Canton, MI).

**Statistical analysis.** Statistical analysis was performed on untransformed ΔC, data, and means were compared by Student’s t-test using PSI-Plot (Pearl River, NY).

**RESULTS**

The Cyp24a1 gene codes for the 25-hydroxyvitamin D (25D₃)-24-hydroxylase enzyme, which is widely expressed throughout the body and thought to be the first enzymatic step in the oxidative process leading to the inactivation of the hormone (1, 50). The protein is found in mucosal cells of the colon (29) and has been reported to be most strongly expressed at the base of the crypts, with diminished expression in the upper parts of the crypts (2). Expression of the gene is strongly enhanced by 1,25D₃ and serves as a sensitive marker for hormone (1, 50). The protein is found in mucosal cells of the colon (29) and has been reported to be most strongly expressed at the base of the crypts, with diminished expression in the upper parts of the crypts (2). Expression of the gene is strongly enhanced by 1,25D₃ and serves as a sensitive marker for ligands capable of binding to and activating the VDR (21), including LNCaP prostate cancer cells (14). To assess the ligands capable of binding to and activating the VDR (21), we then orally administered equimolar amounts (24 pmol) of 1,25D₃ or βGluc-1,25D₃ to C57BL/6 mice and assessed intestinal Cyp24a1 mRNA responses at various time points over the next 24 h. As seen in Fig. 2A, baseline plasma 1,25D₃ concentrations were <0.2 nmol/l. Plasma concentrations of 1,25D₃ rose dramatically 1 h after oral administration of 24 pmol of 1,25D₃, peaking at >2.5 nmol/l; after 9 h, they fell quickly to baseline levels, where they remained. Blood concentrations of 1,25D₃ following oral dosing with the same amount of βGluc-1,25D₃ were modestly elevated through 6 h, peaking at ~1 nmol/l, and then returned to baseline. In the colon (Fig. 2B), βGluc-1,25D₃ profoundly stimulated Cyp24a1 gene expression, as determined by qRT-PCR analysis, with a maximum (~700-fold) increase at 6 h. The same dose of 1,25D₃ also increased colon Cyp24a1 gene expression, but only about fivefold above baseline. The opposite response was failed to enhance Cyp24a1 gene expression and was analogous to the control sample. Thus βGluc-1,25D₃ appears to be stable and not to break down into the free hormone in culture medium for up to 48 h at 37°C, which confirms that the molecule is transcriptionally inert under these conditions (44).

**Statistical analysis.** Statistical analysis was performed on untransformed ΔC, data, and means were compared by Student’s t-test using PSI-Plot (Pearl River, NY).
observed in the duodenum of these mice. Strong stimulation of this gene was observed after only 1 h with 1,25D$_3$ treatment, reaching a maximum (>2,400-fold increase) at 6 h and then receding to control levels. Oral βGluc-1,25D$_3$ increased Cyp24a1 expression in the duodenum, but the magnitude and duration of the gene enhancement were significantly reduced compared with 1,25D$_3$ at various time points (Fig. 2C).

We also examined expression of the epithelial calcium channel transient receptor Trpv6 (42, 48, 54) induced by the vitamin D compounds in digestive tracts of these mice (Fig. 3). After oral administration of βGluc-1,25D$_3$, stimulation of Trpv6 in the mouse colon was maximal at 9 and 12 h and remained modestly elevated through 24 h (Fig. 3A). Treatment with 1,25D$_3$ stimulated colon Trpv6 gene expression at the same time points, but to levels that were less than one-half of those observed with βGluc-1,25D$_3$. Again, the opposite response was observed in the duodenum, where treatment with 1,25D$_3$ resulted in strong, statistically significant enhancement of Trpv6 at 3–12 h, while duodenal induction with the glucuronide was much weaker and only reached statistical significance at 9 and 12 h after treatment (Fig. 3B). These data are consistent with a model whereby the orally administered βGluc-1,25D$_3$ passes through the upper intestinal tract largely intact before the active hormone is released in the lower gut through β-glucuronidase activity associated with resident bacterial populations.

While oral administration of βGluc-1,25D$_3$ was expected to strongly impact gene expression in the lower intestinal tract, we wondered whether the compound would be able to exert activity on the colon if given subcutaneously. We hypothesized that βGluc-1,25D$_3$ would be largely resistant to cleavage by mammalian enzymes while in circulation and, therefore, that its ability to reach the lumen of the colon, where the active hormone could be released by bacterial glucuronidase activity, might be restricted or even eliminated. As seen in Fig. 4A, blood concentrations of 1,25D$_3$ were increased to ~2.0 nmol/l by subcutaneous injection of 1,25D$_3$ and then gradually returned to control levels after 18–24 h. In contrast, injection of βGluc-1,25D$_3$ resulted in a later and smaller rise in serum concentrations of 1,25D$_3$, which peaked at ~0.5 nmol/l and then gradually fell to control levels after 24 h. There was a strong, ~150-fold, increase in Cyp24a1 gene expression in the colon of mice receiving subcutaneous βGluc-1,25D$_3$, which peaked at 9 h, while only weak stimulation of this gene was
observed following subcutaneous injection with 1,25D3 (Fig. 4B). Subcutaneous treatment with 1,25D3 resulted in robust (>200-fold) Cyp24a1 induction in the duodenum of these mice after 6 h, while only weak, transitory stimulation of duodenal Cyp24a1 gene expression was observed with βGluc-1,25D3 (Fig. 4C). The data demonstrate that simply raising plasma concentrations of 1,25D3 did not correlate with enhanced gene expression in the colon and highlight the perplexing lack of responsiveness by the colon to elevated circulating concentrations of 1,25D3 independent of the mode of administration. The data also indicate that our hypothesis that subcutaneous βGluc-1,25D3 would not be active in the colon was incorrect and caused us to suspect that βGluc-1,25D3 was somehow reentering the intestinal tract to release the active hormone in the colon.

In view of these prior results, we hypothesized that subcutaneously administered βGluc-1,25D3 was being removed from the circulation by the liver and being excreted back into the lumen of the upper intestine. To assess the likelihood of this scenario, mice were subjected to a surgical procedure whereby a section of the upper intestine (generally jejunum) was tied off with suture to prevent the passage of digestive contents into the lower colon. Sham-operated mice were included as controls. Mice were then treated with subcutaneous or oral administration of 24 pmol of βGluc-1,25D3 or 1,25D3, and the effects on Cyp24a1 gene expression in the colon were examined 8 h later. As seen in Fig. 5A, subcutaneously or orally administered βGluc-1,25D3 resulted in a significant decrease in Cyp24a1 gene expression, as measured by ΔCt, corresponding to strong induction of gene expression, in the colon of sham-operated mice. However, when the upper intestinal tract was tied off, the ability of βGluc-1,25D3 to stimulate Cyp24a1 gene expression, as evidenced by a decrease in ΔCt activity, was abolished, regardless of mode of administration. As previously observed, induction of Cyp24a1 mRNA following 1,25D3 treatment was greatly muted compared with the βGluc-1,25D3 response in sham-operated mice. Nonetheless, even the weak effect attributable to 1,25D3 treatment, particularly the response to subcutaneous administration, was eliminated when the upper intestine was tied off. Interestingly, surgically tied-off untreated mice also exhibited a significant increase in Cyp24a1 ΔCt activity compared with sham controls. No overt signs of tissue decay were observed over the time period of the experiment for any of the mice, and ΔCt values for the mouse Gapdh gene used in normalizing Cyp24a1 gene expression were statistically the same for all the samples (20.06 ± 0.19 and 19.93 ± 0.17 for sham-operated and surgically tied-off animals, respectively).

To determine whether this response extended to other species, we performed a similar experiment using rats, but the analysis was limited to subcutaneous injections of βGluc-1,25D3 or 1,25D3. As depicted in Fig. 5B, Cyp24a1 gene expression was again strongly enhanced in the colon of sham-operated rats treated with βGluc-1,25D3, as evidenced by the sharp drop in ΔCt values. As in mice, no enhancement of Cyp24a1 gene expression was observed with subcutaneous βGluc-1,25D3 in rat colon when the upper intestinal tract was surgically blocked. Furthermore, treatment with 1,25D3 again produced a very modest increase in gene expression in the colon, which was reduced to control levels when the upper gut was surgically tied off. These data in mice and rats suggest that 1,25D3 and βGluc-1,25D3 are taken up from the circulation and that both compounds are excreted into liver bile.

Finally, Cyp24a1 gene expression was determined in mice fed three different amounts of βGluc-1,25D3 (7.9, 24, and 79 pmol/day) added to the diet for 5 days. As seen in Fig. 6A, at the end of the 5-day treatment period, there was no significant change in plasma 1,25D or calcium concentrations in any animals receiving βGluc-1,25D3 in their diet compared with controls. In contrast, when the results were plotted (Fig. 6B), there was a highly correlative linear relationship (R² = 0.99) between the amount of βGluc-1,25D3 fed to the mice and the response in Cyp24a1 gene expression in the colon of these mice. No statistically significant enhancement of Cyp24a1 gene expression was seen in the corresponding duodenums of the mice (data not shown).

### DISCUSSION

An important aspect of vitamin D metabolism within the colon is the mechanism whereby vitamin D and its metabolites reach this tissue. Classical work on the endocrine action of vitamin D suggests that the active hormone, 1,25D3, is produced in the kidney during negative calcium balance and...
secreted into the general circulation. The circulating 1,25D₃ is then carried to the intestine and thought to cross the basolateral membrane of enterocytes to bind to VDRs. In the duodenum, this results in enhanced calcium and phosphorus transport (7). In the colon, it may affect the propensity for tumor development or inflammatory responses by regulating tight cell junction integrity and local immune function (15, 35).

Another mechanism whereby vitamin D and its metabolites might reach intestinal tissues is excretion of vitamin D metabolites into the bile (25). Both 1,25D₃ and 25D₃ are known to enter the enterohepatic circulation; however, the importance of the enterohepatic circulation of vitamin D compounds to vitamin D deficiency and calcium homeostasis is arguable. Malabsorptive syndromes prevent absorption of vitamin D from the diet but can also lead to loss of endogenous metabolites of vitamin D, leading to osteomalacia associated with bowel disease (49). Clements et al. (8) administered oral and intravenous doses of radioactively labeled vitamin D to patients with T-tube biliary drainage after cholecystectomy. The vitamin was mainly excreted as highly polar inactivation products, and <4% of the metabolites in bile were present as 25D₃ or its glucuronide conjugate. Clements et al. concluded that there was insufficient vitamin D or 25D₃ in the bile for the reabsorption of these metabolites to make a significant contribution to normal vitamin D status. Wiesner et al. (52) administered radiolabeled 1,25D₃ to five normal vitamin D-replete human subjects and found that 15.6% of the injected dose appeared in bile as more polar metabolites of 1,25D₃, most likely in a glucuronidated form. Of the injected dose, 27% and 7.5% appeared in the feces and urine, respectively, at 24 h. In another two subjects, biliary radioactivity was sampled at two jejunal sites separated by a distance of 40 cm; a 24.8% decrease in radioactivity over this segment of bowel suggested that the 1,25D₃ was being reabsorbed as the more polar metabolite or following conversion to the free 1,25D₃ in the intestine. Hashizume et al. (24) also recently demonstrated that the human UDP-glucuronosyltransferase UGT1A4 could efficiently catalyze glucuronidation of 1,25D₃, with the 25-O-glucuronide identified as the major product. Collectively, these data demonstrate that products of vitamin D can be modified by liver cells and excreted in normal human bile and that these products are present in the enterohepatic circulation in normal humans.

The present study establishes the selective effects of βGluc-1,25D₃ on vitamin D-dependent gene expression in the lower colon. Whether the compound was administered orally or subcutaneously, βGluc-1,25D₃ targeted gene expression to the colon of mice and rats, as evidenced by strong stimulation of Cyp24a1 and Trpv6 genes. This was achieved despite much smaller increases in circulating 1,25D₃ concentrations in com-
parison to treatment with the natural hormone. In addition, Cyp24a1 and Trpv6 gene expression in the duodenum were impacted less by βGluc-1,25D₃ than 1,25D₃ administration. This suggests that the majority of the βGluc-1,25D₃ reached the lumen of the lower intestinal tract intact, where it was cleaved by resident bacterial β-glucuronidase enzyme activity to release the active hormone. It also suggests that germ-free animals lacking bacterial cell populations in the intestinal tract should be unresponsive to βGluc-1,25D₃ and presumably demonstrate little or no stimulation of vitamin D-dependent gene expression in the colon.

The current experiments reveal that the bulk of 1,25D₃ given to mice acted on upper intestinal cells and that only minute amounts appeared to reach the colon. Administration of 24 pmol of 1,25D₃ produced robust stimulatory responses for Cyp24a1 and Trpv6 gene products in the duodenum. However, the colon was relatively unaffected by treatment with the natural hormone, as very little stimulation of these gene products was observed, despite significantly higher circulating concentrations of 1,25D₃. Our findings suggest that 1,25D₃ reaching the basolateral surface of the colonic epithelium via the circulation is less able to stimulate vitamin D-dependent gene expression than apically delivered hormone. The data further question the concept that circulating 1,25D₃ is free to diffuse into and out of cells and suggest that cell polarity and possible active transport systems may be involved in the hormone’s cellular entry. Moreover, the data highlight the difficulties in trying to establish doses of 1,25D₃ that inhibit in vivo colon tumor formation or growth. Our findings suggest that it will be virtually impossible to administer doses of 1,25D₃ that are high enough to produce concentrations of hormone in the colon of test subjects sufficient to inhibit tumorigenesis without also causing severe hypercalcaemia. Our data also imply that, in many of the previous studies examining 1,25D₃ or its analogs as colon cancer therapeutic agents, only the effects of a small fraction of the administered compounds that escaped absorption or reabsorption to reach the colon were seen (13, 30, 38, 53).

The inability of βGluc-1,25D₃ or 1,25D₃ to alter gene expression when passage of digestive contents to the colon was blocked confirms these concepts and leads us to the following conclusions. 1) Orally administered βGluc-1,25D₃ reaches the lumen of the lower intestinal tract, where it is cleaved and delivers 1,25D₃ to the apical membrane of colon cells, resulting in strong upregulation of vitamin D-dependent genes. 2) Subcutaneously administered βGluc-1,25D₃ in mice and rats only reaches the colon via passage of ingesta from the upper to the lower intestinal tract. This observation is consistent with the hypothesis that the liver may be removing βGluc-1,25D₃ from the circulation and excreting it, most likely still in the β-glucuronide form, into the duodenum, where it flows with the ingesta to the colon and is cleaved. 3) While oral 1,25D₃ failed to significantly upregulate colon Cyp24a1 in the mouse study, subcutaneous administration of the same dose caused a slight upregulation of colon gene expression in mice and rats. However, when the flow of digesta to the colon was blocked by tying off the small intestine of mice and rats, the weak Cyp24a1 response to subcutaneous 1,25D₃ treatment was also abolished, although the change was statistically significant only for mice (Fig. 5). This suggests that even the low level of activity attributable to subcutaneously delivered 1,25D₃ is mediated by entry into the lumen of the gut and its crossing of the apical membrane of colon cells. Furthermore, basal Cyp24a1 activity in the untreated mice also dropped significantly when the upper digestive tract was blocked, and a similar trend was exhibited in rats (Fig. 5). Whether endogenous 1,25D₃ is excreted into the bile to reach the lower colon or some portion of the circulating hormone is taken up and modified by the liver to a glucuronide form prior to excretion into bile, which is then liberated in the colonic lumen, remains to be elucidated. However, the x-intercept of the best-fit line from the dose-response data (Fig. 6) suggests that basal expression of Cyp24a1 in the colon of untreated, vitamin D-replete control mice corresponds to exposure to the equivalent of ~2–4 pmol/day of 1,25D₃ activity. Other factors could be involved in determining the weak basal expression of Cyp24a1 in the colon of these mice, but this estimate is consistent with the observations from untreated animals in the surgical tie-off experiments (Fig. 5) and other reports that liver cells can modify 1,25D₃ and excrete a glucuronidated form into bile (24, 36).

In prior work from our laboratory, it was noted that feeding of βGluc-1,25D₃ for up to 8 days produced only a slight rise in blood calcium concentrations, while the same dose of 1,25D₃ yielded a significant rise in plasma calcium (20). It is clear that βGluc-1,25D₃ does not raise blood 1,25D₃ concentrations as much as the native hormone. It is also has a far less potent effect on duodenal gene expression and would be less likely to activate calcium absorption mechanisms than the native hormone. This would seem to be borne out by observations in our earlier report (20) and in the current study, which found that feeding variable amounts of βGluc-1,25D₃ caused no significant rise in blood calcium concentrations. Accordingly, βGluc-1,25D₃ appears to satisfy two of the requirements that would warrant further study for the treatment of colon tumors or IBD: 1) it selectively targets the colon, as evidenced by gene expression, and 2) it does not elicit a significant rise in blood calcium concentrations.

The importance of hepatic excretion of vitamin D compounds and the enterohepatic circulation of vitamin D compounds, particularly 1,25D₃, to normal vitamin D physiology was largely dismissed, because duodenal activity and calcium metabolism did not seem to be greatly affected by the hepatic metabolism. However, our study suggests that this phenomenon is more than a physiological curiosity or simple way of disposing of vitamin D from the body; it is likely the predominant means whereby active forms of vitamin D are delivered to the colonic epithelium. Moreover, our study suggests a commensal relationship with resident bacterial populations in the lower gut that liberate vitamin D metabolites passing down the lumen of the colon. In this way, the apparently more responsive apical membrane of host colonic epithelia or immune cells can absorb the freed metabolites to carry out their cellular functions. This may be a critical pathway in maintaining overall colon health in an area of the digestive tract that would otherwise be lacking ready access to the vitamin. More recent discoveries focused on the autocrine action of vitamin D (3, 10) suggest that the 1,25D₃ important to colon function could also arise from colon tissue 1α-hydroxylase action on 25D₃. Therefore, further investigation is also warranted to determine whether the known glucuronidation of 25D₃ in the liver and its secretion into bile could be a means of targeting release of 25D₃ in the colon to provide substrate for local 1,25D₃ production. This scenario
could provide a further link between the vitamin D status of an individual, as determined by circulating 25D₃ concentrations, and colon health, as suggested by numerous epidemiological studies (12, 16, 18, 26, 31, 37, 40).

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DISCLOSURES

Glycomyry (Ames, IA) supplied the bGlu-1,25D₃ used in the studies. J. P. Goff and R. L. Horst are president and vice-president, respectively, of this company, which is working to develop commercial uses of glycosides of vitamin D.

AUTHOR CONTRIBUTIONS

N.J.K., R.L.H., and J.P.G. are responsible for conception and design of the research; N.J.K., R.L.H., and J.P.G. performed the experiments; N.J.K., R.L.H., and J.P.G. analyzed the data; N.J.K., R.L.H., and J.P.G. interpreted the results of the experiments; N.J.K. prepared the figures; N.J.K. drafted the manuscript; N.J.K., R.L.H., and J.P.G. edited and revised the manuscript; N.J.K., R.L.H., and J.P.G. approved the final version of the manuscript.

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