Temporal changes in tissue 1α,25-dihydroxyvitamin D₃, vitamin D receptor target genes, and calcium and PTH levels after 1,25(OH)₂D₃ treatment in mice

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Chow EC, Quach HP, Vieth R, Pang KS. Temporal changes in tissue 1a,25-dihydroxyvitamin D₃, vitamin D receptor target genes, and calcium and PTH levels after 1,25(OH)₂D₃ treatment in mice. Am J Physiol Endocrinol Metab 304: E977–E989, 2013. First published March 12, 2013; doi:10.1152/ajpendo.00489.2012.—The vitamin D receptor (VDR) maintains a balance of plasma calcium and 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], its natural active ligand, by directly regulating the calcium ion channel (TRPV6) and degradation enzyme (CYP24A1), and indirectly regulating the parathyroid hormone (PTH) for feedback regulation of the synthetic enzyme CYP27B1. Studies that examined the intricate relationships between plasma and tissue 1,25(OH)₂D₃ levels and changes in VDR target genes and plasma calcium and PTH are virtually nonexistent. In this study, we investigated temporal correlations between tissue 1,25(OH)₂D₃ concentrations and VDR target genes in ileum and kidney and plasma calcium and PTH concentrations in response to 1,25(OH)₂D₃ treatment in mice (2.5 µg/kg ip, singly or q2d × 4). After a single ip dose, plasma 1,25(OH)₂D₃ peaked at ~0.5 h and then decayed biexponentially, falling below basal levels after 24 h and then returning to baseline after 8 days. Upon repetitive ip dosing, plasma, ileal, renal, and bone 1,25(OH)₂D₃ concentrations rose and decayed in unison. Temporal profiles showed increased expressions of ileal Cyp24a1 and renal Cyp24a1, Mdr1/P-gp, and VDR but decreased renal Cyp27b1 mRNA after a time delay in VDR activation. Increased plasma calcium and attenuated PTH levels and increased ileal and renal Trpv6 expression paralleled the changes in tissue 1,25(OH)₂D₃ concentrations. Gene changes in the kidney were more sustained than those in intestine, but the magnitudes of change for Cyp24a1 and Trpv6 were lower than those in intestine. The data revealed that 1.25(OH)₂D₃ equilibrates with tissues rapidly, and VDR target genes respond quickly to exogenously administered 1.25(OH)₂D₃.

1α,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃] is the active form of vitamin D and natural ligand of the vitamin D receptor (VDR), a member of the steroid/thyroid hormone nuclear receptor superfamily (34). Vitamin D is formed from 7-dehydrocholesterol in skin upon exposure to the ultraviolet rays of the sun (270–300 nm) and is hydroxylated by the cytochrome P-450s (CYP2R1 and CYP27A1) in liver to form 25-hydroxyvitamin D₃ [25(OH)D₃]. This inactive metabolite is transported by the vitamin D binding protein (DBP) and taken up by the endocytic receptor megalin into renal proximal tubular cells (41, 48) for activation by the 1α-

hydroxylase (CYP27B1) to form the active metabolite, 1,25(OH)₂D₃ (30, 49).

Upon binding of 1,25(OH)₂D₃ to VDR, the complex undergoes a conformational change and translocates to the nucleus to heterodimerize with the retinoid X receptor (RXR) (4, 32), followed by recruitment of coactivators before binding to vitamin D response elements (VDREs) in promoter regions of VDR-responsive genes to initiate gene transcription (19). One of the physiological roles of 1,25(OH)₂D₃ is to increase plasma calcium levels through transactivation of the calcium ion channels [transient receptor potential cation channel subfamily V members 5 and 6 (TRPV5 and TRPV6)] in the kidney and intestine (46). It is known that calcium is maintained by the concerted actions in not only the epithelia of the kidney and intestine, but also bone, where turnover is a continuous process involving both resorption of existing bone and deposition of new bone, processes that are stimulated by actions of 1,25(OH)₂D₃ and the parathyroid hormone, PTH (28, 30).

The level of 1,25(OH)₂D₃ in plasma is tightly controlled by two major cytochrome enzymes in the kidney, CYP27B1 or 1α-hydroxylase, which converts 25(OH)D₃ to 1,25(OH)₂D₃, and CYP24A1, the catabolic enzyme that degrades 25(OH)D₃ and 1,25(OH)₂D₃ to 24,25(OH)₂D₃ and 1,24,25(OH)₃D₃, respectively (23). CYP24A1 is present in tissues that express VDR (3) and serves as a biomarker for VDR activation. When 1,25(OH)₂D₃ in plasma is high, CYP24A1 becomes highly induced in the kidney to increase catabolism (36). When the circulating calcium concentration is low, the parathyroid gland responds by stimulating the production of PTH to downregulate CYP24A1 mRNA stability in kidney, reducing 1,25(OH)₂D₃ degradation to result in higher plasma levels of 1,25(OH)₂D₃ (56, 57). When plasma calcium level is high, activation of the calcium-sensing receptor (CaSR) leads to reduction of PTH (23, 30). In the intestine, the CYP24A1 level is regulated by 1,25(OH)₂D₃ and not PTH (45), suggesting that induction of CYP24A1 in the intestine is more of an acute response to VDR than the kidney (26).

Within the past decade, the VDR has been implicated to play an important role in the regulation of drug enzymes and transporters. VDR-responsive drug-metabolizing enzymes include the cytochrome P-450s [human CYP3A4, CYP2B6, and CYP2C9 (17, 50), and rodent Cyp3a1, Cyp3a9, and Cyp2a11 (12, 14)] and sulfotransferase-2A1 (SULT2A1) (20). VDR-responsive transporters include the rat apical sodium-dependent bile acid transporter (Asbt) (10), human organic anion-transporting polypeptide (OATP1A2) (21), multidrug resistance protein-1 or P-glycoprotein (MDR1/P-gp) (44), and the human and rodent multidrug resistance-associated proteins (MRP2/Mrp2, Mrp3, MRP4/Mrp4) both in vitro and in vivo.

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Our laboratory has shown that VDR transactivates P-gp in brain microvessel endothelia (18) in vitro and P-gp in murine kidney and brain but not ileum and liver in vivo, leading to hastened efflux of digoxin in the brain and kidney (11).

Clinically, the concentration of 25(OH)D3 in plasma (nM range) is used as the biomarker for vitamin D status, although this value does not reflect the concentration of its active metabolite, 1,25(OH)2D3 (pM range) formed via CYP27B1, the rate-limiting enzyme (19, 53). Thus, there is the need to define precisely the pharmacological effects and interplay between 1,25(OH)2D3 and the respective VDR target genes. A temporal study was thus undertaken to examine tissue levels of doses of 1,25(OH)2D3 (2.5 g/kg or 38 mg/mouse or 120 mg/kg) furnished a similar clearance and exposure with the immunocapsules for EIA.

Preparation of subcellular protein fractions of kidney and intestinal tissues. For preparation of the crude membrane fraction for the assay of Cyp24 or P-gp protein, kidney, colon tissue, or scraped enterocytes were homogenized in the crude membrane homogenizing buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Trizma base, pH 7.4) containing 1% protease inhibitor cocktail (11). This homogenate was diluted 350-fold with 1% nitric acid before each measurement. The mithylene chloride (bottom phase) was collected using a glass Pasteur pipette. Extraction of the homogenate was repeated upon addition of 1.25 ml of methylene chloride before the bone mixture was crushed using a mortar and pestle to obtain a homogenate. The homogenate was then centrifuged at 3,000 × g for 10 min through a 0.2 µm Nanosep MF Centrifugal Device (Pall Life Sciences, Ville St. Laurent, QC) before addition for the immunocapsules for EIA.

The supernatant was spun at 20,000 × g for 10 min to 5 ml of a mixture of methylene chloride and methanol (1:2 vol/vol) before analysis. Delipidated solutions of tissue samples were spun at 12,000 × g for 10 min through a 0.2 µm Nanosep MF Centrifugal Device (Pall Life Sciences, Ville St. Laurent, QC) before addition for the immunocapsules for EIA.

Preparation of subcellular protein fractions of kidney and intesti- nal tissues. For preparation of the crude membrane fraction for the assay of Cyp24 or P-gp protein, kidney, colon tissue, or scraped enterocytes were homogenized in the crude membrane homogenizing buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Trizma base, pH 7.4) containing 1% protease inhibitor cocktail (11). This homogenate was used for Western blotting to measure total VDR protein expression. The homogenate was then centrifuged at 3,000 × g for 10 min at 4°C, and the resulting pellet containing the mitochondria was resuspended in a buffer (in nM: 60 KCl, 15 NaCl, 5 MgCl2·6H2O, 0.1 EGTA, 300 sucrose, 0.5 DTT, 0.1 PMSF, 300 sucrose, and 15 Trizma HCl, pH 7.4) containing 1% protease inhibitor cocktail for Western blotting to measure Cyp24 protein expression. The supernatant was spun at 20,000 × g for 60 min at 4°C, and the resulting pellet was resuspended in the resuspension buffer, as previ-
plasma binding of 1,25(OH)2D3 relative to those in tissues. The less-than-unity tissue partitioning ratio may be explained by the high plasma concentration ratios were 0.35, 0.4, and 0.17 for the
B
pmol/kg tissue), and bone (36.5 pmol/kg tissue). Basal levels of 1,25(OH)2D3 in the
A
significantly lower than those in plasma (Fig. 1
A
); the tissue/
B
plasma concentration (Cmax) of 44 nM, followed by an apparent, biexponential decay profile (Fig. 2
A
) with a half-life of ~6.86 h (Table 2). Distinctively, the plasma concentration of 1,25(OH)2D3 fell below the baseline by the end of day 0, reached a nadir by day 4, and returned to baseline levels by day 8 (Fig. 2
A
). Subsequent to ip dosing of 1,25(OH)2D3, tissue levels in the kidney, ileum, and bone peaked at similar times [6, 4.5, and 0.065 mmol/kg (or pmol/g) tissue, respectively] and decayed in parallel fashion to that of plasma (Fig. 2
A
).

Following multiple 1,25(OH)2D3 ip dosing, patterns of decay for plasma 1,25(OH)2D3 were similar after each injection, wherein the 1,25(OH)2D3 concentration peaked (averages of 44, 22, 43, and 29 nM) between 0.5 and 1 h after each of the injections given. Levels again fell below the basal level by the end of 24 h postinjection (Fig. 2
B
). The plasma 1,25(OH)2D3 concentration before the next injection at the nadir was much

The baseline plasma concentration of 1,25(OH)2D3 for vehicle-treated C57BL/6 mouse, estimated as the mean of the determinations for the experimental duration, was 212 ± 29 pM (fmol/ml), a value similar to the endogenous plasma concentration of 1,25(OH)2D3 in the rat (51) but higher than that in humans (8, 53). Basal levels of 1,25(OH)2D3 in the kidney (70.5 ± 10.4 pmol/kg tissue), intestine (93.5 ± 7.2 pmol/kg tissue), and bone (36.5 ± 33.8 pmol/kg tissue) were significantly lower than those in plasma (Fig. 1A); the tissue/plasma concentration ratios were 0.35, 0.4, and 0.17 for the kidney, ileum, and bone, respectively (Fig. 1B). The less-than-unity tissue partitioning ratio may be explained by the high plasma binding of 1,25(OH)2D3 relative to those in tissues.

### Table 1. Mouse primer sets for quantitative real-time PCR

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<td>CTCACGCTCGTATCGACGC</td>
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<tr>
<td>Mdr1</td>
<td>TACGACGCCATATCTGACTG</td>
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<td>Trpv6</td>
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<td>ACCCTGTTCCTGCTGGATGG</td>
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<td>GCCGGATGCTGCTGAGTT</td>
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<tr>
<td>Villin</td>
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Cyp24a1, degradation enzyme; Mdr1, multidrug resistance protein-1; Trpv6, calcium ion channel; VDR, vitamin D receptor.

### RESULTS

**Similar plasma and tissue (ileum, kidney, and bone) decay of 1,25(OH)2D3 after single and multiple dosing of 1,25(OH)2D3 to mice.** The baseline plasma concentration of 1,25(OH)2D3 for vehicle-treated C57BL/6 mouse, estimated as the mean of the determinations for the experimental duration, was 212 ± 29 pM (fmol/ml), a value similar to the endogenous plasma concentration of 1,25(OH)2D3 in the rat (51) but higher than that in humans (8, 53). Basal levels of 1,25(OH)2D3 in the kidney (70.5 ± 10.4 pmol/kg tissue), intestine (93.5 ± 7.2 pmol/kg tissue), and bone (36.5 ± 33.8 pmol/kg tissue) were significantly lower than those in plasma (Fig. 1A); the tissue/plasma concentration ratios were 0.35, 0.4, and 0.17 for the kidney, ileum, and bone, respectively (Fig. 1B). The less-than-unity tissue partitioning ratio may be explained by the high plasma binding of 1,25(OH)2D3 relative to those in tissues.

After a single ip dose of 50 ng/mouse (120 pmol/mouse or 2.5 µg/kg), 1,25(OH)2D3 was rapidly absorbed (tmax or time for maximum concentration of ≤0.5 h), yielding a peak plasma concentration (Cmax) of 44 nM, followed by an apparent, biexponential decay profile (Fig. 2A) with a half-life of ~6.86 h (Table 2). Distinctively, the plasma concentration of 1,25(OH)2D3 fell below the baseline by the end of day 0, reached a nadir by day 4, and returned to baseline levels by day 8 (Fig. 2A). Subsequent to ip dosing of 1,25(OH)2D3, tissue levels in the kidney, ileum, and bone peaked at similar times [6, 4.5, and 0.065 mmol/kg (or pmol/g) tissue, respectively] and decayed in parallel fashion to that of plasma (Fig. 2A).

Following multiple 1,25(OH)2D3 ip dosing, patterns of decay for plasma 1,25(OH)2D3 were similar after each injection, wherein the 1,25(OH)2D3 concentration peaked (averages of 44, 22, 43, and 29 nM) between 0.5 and 1 h after each of the injections given. Levels again fell below the basal level by the end of 24 h postinjection (Fig. 2B). The plasma 1,25(OH)2D3 concentration before the next injection at the nadir was much
Fig. 2. Plasma and tissue 1,25(OH)$_2$D$_3$ (ileum, kidney, and bone) concentration-time profiles from a single dose (A) or multiple doses (B) (days 0, 2, 4, and 6) of 2.5 μg/kg ip 1,25(OH)$_2$D$_3$ q2d × 4 to mice. Data for vehicle-treated mice (control) were averaged and are denoted as open circles interconnected by solid line (n = 2–4). For treated mice, individual 1,25(OH)$_2$D$_3$ datum is shown (filled circle, one mouse per sample); averaged values are joined by dashed line (n = 2–4).
lower than that of the basal level, and the same pattern persisted throughout the dosing regimen. The renal, ileal, and bone 1,25(OH)2D3 tissue concentrations rose in unison to those in plasma, reaching a peak concentration of 6.1, 1.8, 5.0, and 3.6 nmol/kg tissue in kidney, 4.5, 1.9, 1.9, and 2.8 nmol/kg tissue in ileum, and 0.065, 0.505, 7.65, and 1.8 nmol/kg tissue in bone, respectively, between 0.5 and 3 h after each of the four injections. These data confirm that 1,25(OH)2D3 is able to equilibrate readily between plasma and tissue.

Multiple dosing of 1,25(OH)2D3 resulted in a terminal or beta half-life of ~6.3 to 7.5 h (Fig. 2B), and no trend was discernable upon repeated dosing (Table 2). Overall, the decay pattern of 1,25(OH)2D3, based on the total 1,25(OH)2D3 (exogenous + basal) concentration after the administered 1,25(OH)2D3, and the exposure (AUC0-48) were similar after each injection (Fig. 2B, Table 2). There was little change in the pharmacokinetics of 1,25(OH)2D3 upon repeated dosing, since change in the enzyme for catabolism, Cyp24a1, was maximal after a single dose (see results below for intestine and kidney). The half-life is similar to that observed from other ip studies in the mouse (7.6 h) (37).

**Intestinal distribution and effects of 1,25(OH)2D3 on intestinal and colon VDR, Cyp24a1, and Trpv6 mRNA expression.** The distribution of basal VDR mRNA expression was found to be higher for the duodenum than jejunum (50% of duodenum) and ileum (46% of duodenum) but was highest in the colon (1.5-fold of duodenum; Fig. 3A, left). Basal Cyp24a1 mRNA

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Table 2. Noncompartmental estimates for 1,25(OH)2D3, after repeated doses of 2.5 µg/kg q2d × 4 ip to mice

<table>
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<th>Dose</th>
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<td>120</td>
<td>120</td>
<td>120</td>
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<td>Terminal decay constant, β, h⁻¹</td>
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<td>0.09</td>
<td>0.10</td>
<td>0.11</td>
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<tr>
<td>AUC0-48, nM × h⁻¹</td>
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<td>131</td>
<td>170</td>
<td>117</td>
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</tbody>
</table>

*Terminal decay constant (β) was estimated from the negative slope of ln(concentration) vs. time data from averaged data between 12 and 48 h after ip dosing. **Terminal half-life (t½) was calculated as 0.693/β. *Area under the curve [AUC0–48] between 0 and 48 h was estimated by the trapezoidal rule.

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Fig. 3. Intestinal distribution of mRNA and effect of 1,25(OH)2D3 (left) on vitamin D receptor (VDR; A), degradation enzyme (Cyp24a1; B), and calcium ion channel (Trpv6; C) mRNA expression in duodenum, jejunum, ileum, and colon at 3 h post-2.5 µg/kg ip 1,25(OH)2D3 injection; temporal changes for ileal mRNA of VDR (A), Cyp24a1 (B), and Trpv6 (C) after a single dose (middle) or multiple doses (right) of 1,25(OH)2D3. Data at left represent mean ± SE (n = 3 or 4).

In left, †p < 0.05 between basal duodenal control vs. basal control of other intestinal segments. *p < 0.05, basal control vs. 1,25(OH)2D3-treated group (Mann-Whitney U-test). In middle and right, data for vehicle-treated mice (control) were averaged and are denoted as open circles interconnected by solid line (n = 2–4). For treated mice, individual 1,25(OH)2D3 datum is shown (filled circle); averaged values are joined by dashed line (n = 2–4).
Fig. 4. Temporal changes in renal mRNA expression of VDR (A), Cyp24a1 (B), Trpv6 (C), and synthetic enzyme, Cyp27b1, (D) after a single dose or multiple doses of 1,25(OH)2D3 to mice. Data for vehicle-treated mice (control) were averaged and are denoted as open circles interconnected by solid line (n = 2–4). For treated mice, individual 1,25(OH)2D3 datum is shown (filled circle); averaged values are joined by dashed line (n = 2–4).
expression was evenly distributed in the small intestine but was highest in colon (42-fold of duodenum; Fig. 3B, left). The basal mRNA expression of Trpv6 was highest in the duodenum, followed by the colon (46% of duodenum), and was negligible in the jejunum and ileum (<1%; Fig. 3C, left). At 3 h post-1,25(OH)₂D₃ injection, there was no major change in VDR mRNA expression for all intestinal segments and the colon (Fig. 3A, left). By contrast, Cyp24a1 and Trpv6 mRNA expressions were elevated >900-fold and >7-fold in the duodenal and ileal segments, respectively, although not for the jejunum due to sample variation (Figs. 3, B and C, left). The lack of Cyp24a1 mRNA induction and small Trpv6 mRNA change in colon (Figs. 3, B and C, left) with 1,25(OH)₂D₃ treatment agrees with the possibility that very low amounts of 1,25(OH)₂D₃ are available to enter into colon due to the route of administration (ip).

Focusing on the ileum, where induction of Asbt (an important VDR target gene to transport bile acids) was previously found to occur in the rat (10, 12), single or repetitive treatment of 1,25(OH)₂D₃ to mice elicited only minimal changes in ileal VDR mRNA expression (Fig. 3A, middle and right), though the temporal ileal 1,25(OH)₂D₃ concentrations rose in unison to that in plasma and remained mostly above basal levels during each injection interval (Fig. 2). By contrast, ileal Cyp24a1 mRNA was induced significantly between 3 and 9 h after dosing (Fig. 3B, middle and right), with patterns similar to the temporal changes of 1,25(OH)₂D₃ in ileum (Fig. 2). The induction of Cyp24a1 mRNA was dramatically increased (>500-fold) after the first dose (Fig. 3B, middle), but the inductions were slightly lessened (300- to 400-fold) for the 2nd, 3rd, and 4th doses, with mRNA levels rapidly returning to baseline at 24 h after dosing (Fig. 3B, right). A single administration of 1,25(OH)₂D₃ resulted in a 30-fold maximal increase in ileal Trpv6 mRNA at 9 h (Fig. 3C, middle). However, multiple dosing of 1,25(OH)₂D₃ greatly magnified the increase of ileal Trpv6 to 200- to 600-fold, with higher changes observed at the 3rd and 4th doses (Fig. 3C, middle and right).

Induction of renal VDR, Cyp24a1, and Trpv6 and down-regulation of Cyp27b1 mRNA were time and concentration dependent. Unlike the modest change of VDR mRNA observed for the intestine, renal VDR mRNA rose 2-fold between 3 and 12 h after the single 1,25(OH)₂D₃ dose, and this increase was sustained for the entire week before levels returned to baseline. With multiple dosing, renal VDR mRNA levels for subsequent doses were further increased to 2.5- 4-fold higher over basal levels (Fig. 4A). After a single 1,25(OH)₂D₃ dose, renal Cyp24a1 mRNA expression was increased 77-fold and peaked at ~9 h, and levels remained high and above baseline for 8 days, whereas multiple dosing of 1,25(OH)₂D₃ induced and maintained Cyp24a1 mRNA over 40-fold above basal levels (Fig. 4B). Injection of a single 1,25(OH)₂D₃ dose resulted in a 5-fold maximal increase in renal Trpv6 mRNA at 9 h, whereas multiple dosing greatly magnified the increase of renal Trpv6, with higher changes observed for Trpv6 mRNA at the 3rd and 4th doses of ~10- to 12-fold above basal levels (Fig. 4C). For renal Cyp27b1 mRNA, there was an immediate rise above basal level at 0.5 h following the first injection, but this change was followed by a rapid decrease to 34% of basal level at 3 h (Fig. 4D); these levels were maintained below basal levels (8–30%) before returning back to baseline on the 8th day, and the average renal Cyp27b1 mRNA level was ~3–30% of basal levels during repetitive dosing (Fig. 4D), suggesting that endogenous 1,25(OH)₂D₃ synthesis was likely reduced.

Induction of renal Mdr1 mRNA and P-gp protein by 1,25(OH)₂D₃. Similarly, renal Mdr1 mRNA showed a relatively small increase (1.5- to 2-fold) before returning back to basal level 6 h after the single administration (Fig. 5A). However, upon repetitive dosing, a disproportionate and sustained increase (>10-fold) was observed (Fig. 5B). Renal P-gp protein levels resulting from 1,25(OH)₂D₃ repetitive treatment rose on average 5-fold above basal levels (Fig. 5C).

Temporal changes in intestinal and renal VDR, Cyp24, and Trpv6 protein expression vs. plasma calcium and PTH levels in single and multiple doses of 1,25(OH)₂D₃ in mice. Basal protein level of VDR was higher in the duodenum than in ileum (30% of duodenum) but was highest in colon (4-fold; Fig. 6A), whereas VDR protein was similar between the duodenum and kidney (Fig. 6B). Changes in protein expression of VDR were different compared with its mRNA expression (Fig. 3A). When examined, VDR protein in the various segments showed differential temporal changes with 1,25(OH)₂D₃ treatment for the duodenum, ileum, and colon. VDR protein in duodenum was elevated after the 2nd and 3rd 1,25(OH)₂D₃ injections, whereas VDR protein level in the colon was increased after the 3rd and 4th injections (Fig. 6C). There were higher protein changes but a lack of change in ileal VDR.
mRNA with treatment (Fig. 3A), and this could be explained by the action of 1,25(OH)2D3 in increasing the half-life of VDR protein (31). Renal VDR protein, similar to VDR mRNA, rose rapidly after the 1st dose, and was sustained for the 2nd and 4th doses (Fig. 6C). Furthermore, multiple administration of 1,25(OH)2D3 increased Cyp24 protein in ileum steadily at 1.5-fold above basal level (Fig. 7A), whereas the changes for renal Cyp24 protein were considerably higher (10-fold on average) than in ileum (Fig. 7B).

We then examined basal levels of Trpv6 protein in the duodenum, ileum, and colon and found the rank order of duodenum > ileum (72% of duodenum) > colon (25% of duodenum) (Fig. 8A). The basal level of Trpv6 protein of the kidney was only 43% of that in duodenum (Fig. 8B). With 1,25(OH)2D3 treatment, Trpv6 protein levels were increased 1.5-fold on average throughout the treatment period for the duodenum and ileum after the 3rd and 4th doses, whereas levels in the colon were increased after the 4th dose (Fig. 8C). These changes in

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**Fig. 6.** Distribution (A and B) and temporal changes (C) of VDR protein (54 kDa) in duodenum, jejunum, ileum, colon, and kidney after multiple doses of 1,25(OH)2D3. A: comparison of VDR protein in different intestinal segments was normalized to villin. B: comparison of VDR protein between duodenum and kidney was normalized to Gapdh. Data in A and B represent means ± SE (n = 3 or 4), A: † P < 0.05, basal duodenal control vs. basal control of other intestinal segments (Mann-Whitney U-test). C: data for vehicle-treated mice (control) were averaged and are denoted as open circles interconnected by solid line (n = 2–4). For treated mice, individual 1,25(OH)2D3 datum is shown (filled circle); averaged values are joined by dashed line (n = 2–4).

**Fig. 7.** Temporal changes in ileal (A) and renal (B) relative protein expression of Cyp24 (55 kDa) after multiple doses of 1,25(OH)2D3. Data for vehicle-treated mice (control) were averaged and are denoted as open circles joined by solid line (n = 2–4). For treated mice, individual 1,25(OH)2D3 datum is shown (solid circle); averaged values are joined by dashed line (n = 2 to 4).
protein are consistent with a high Trpv6 mRNA induction in ileum at the same period (Fig. 3C).

Furthermore, levels of plasma calcium were correlated to changes in Trpv6 mRNA (Fig. 3C) and protein (Fig. 8C). There was virtually no discernable change in calcium levels after a single dose, whereas cumulative changes were observed after the 2nd to 4th doses (Fig. 8D). These changes could be attributed to the relatively high and transient elevation of both the intestinal and renal Trpv6 mRNA, raising the plasma calcium concentrations by 10 – 40% during the successive dosing regimen (Fig. 8D). However, due to the 2.2-fold higher Trpv6 protein level in the duodenum compared with that in kidney (Fig. 8B), the intestine is likely a greater contributor to calcium absorption. This notion was supported by others as well (15, 54).

The mean basal plasma PTH level was \(68.8 \pm 14.8\) pg/ml in control mice (Fig. 9). After a single dose of 1,25(OH)\(_2\)D\(_3\), mouse plasma PTH initially increased (to \(\sim 148\) pg/ml) in the first 5 min postinjection, but levels then immediately dropped to 12–34 pg/ml between 6 and 48 h before returning back to basal levels on the eighth day (Fig. 9A). Multiple dosing of 1,25(OH)\(_2\)D\(_3\) to mice generally led to sustained decreased plasma PTH (between 0 and 30 pg/ml) throughout the course of treatment, except at two sampling time points (0.5 h after 3rd injection and 9 h after 4th injection; Fig. 9B), likely due to sampling variation and small sample size.

**DISCUSSION**

Our efforts represent one of the first studies to examine plasma and tissue 1,25(OH)\(_2\)D\(_3\) concentrations accompanying the exogenous 1,25(OH)\(_2\)D\(_3\) administration to mice. Despite being tightly bound to the vitamin D binding protein (DBP) in the plasma, we observed rapid distribution of 1,25(OH)\(_2\)D\(_3\) into tissues due to the lipophilic nature of the compound (19). 1,25(OH)\(_2\)D\(_3\) is able to enter and distribute into tissues rapidly, including the kidney, intestine, bone (Fig. 1), liver, and brain (data not shown) regardless of differences in VDR abundance. The parallel patterns of rise and decay for 1,25(OH)\(_2\)D\(_3\) sug-
gest rapid entry, distribution, and equilibrium between tissue and plasma. 1,25(OH)₂D₃ concentrations in the kidney and intestine remained predominantly above basal levels during and after 1,25(OH)₂D₃ treatment (Fig. 2), implying that the present regimen for treatment of 1,25(OH)₂D₃ could result in sustained local pharmacological effects. Under basal conditions, the tissue-to-plasma partitioning ratios are for ileum 0.41 ± 0.12, for kidney 0.33 ± 0.07, and for bone 0.15 ± 0.16 (Fig. 1). Those in the liver (0.13 ± 0.04) and brain (0.007 ± 0.003) were also lower (data not shown).

Upon correlation of the plasma and tissue 1,25(OH)₂D₃ concentration-time profiles to changes of VDR target genes in mice, we found that maximal induction of VDR target genes such as Trpv6 and Cyp24a1 mRNA expression in intestine were similar after single vs. repeated dosing (Figs. 3, B and C), with the peak occurring between 3 and 9 h postinjection, lagging behind the peak 1,25(OH)₂D₃ concentration in ileum (at ~0.5–1 h) (Fig. 2). This lag time is not unexpected and is likely the result of the time required for translocation of the VDR into the nucleus for heterodimerization with the RXR to initiate transcription. Changes in renal VDR, Cyp24a1, and Mdr1 mRNA expression also showed the time lag but were more sustained after repeated dosing, since the VDR level was elevated and was more responsive to 1,25(OH)₂D₃ treatment (Figs. 4 and 5). In addition, tissue ileal and kidney concentrations of 1,25(OH)₂D₃ at 3–9 h after each 1,25(OH)₂D₃ administration remained elevated above baseline values (Fig. 2), at which time maximal induction of renal and ileal Cyp24a1, VDR and Trpv6 mRNA expression was noted (Figs. 3 and 4).

Cyp24a1 is a major VDR-responsive gene (9, 29) that metabolizes 1,25(OH)₂D₃ to 1,24,25-trihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ to 24,25-dihydroxyvitamin D₃ (30), and absence of Cyp24a1 in Cyp24 knockout mice drastically reduces 1,25(OH)₂D₃ metabolism (35). In this study, we found that induction of ileal Cyp24a1 mRNA (Fig. 3B) was much greater than that for renal Cyp24a1 (Fig. 4B), although renal Cyp24a1 induction was more sustained (maintained above 40-fold of basal level) than that of the ileum, whose Cyp24a1 levels rapidly returned to basal levels at 24 h (Fig. 3B). There are perhaps two potential explanations. First, enterocytes in the intestine have a much higher turnover rate than renal tubular cells (7, 24), reducing the sustainability of ileal Cyp24a1 induction. Second, renal and not ileal VDR was induced, and renal induction of VDR continued upon multiple dosing of 1,25(OH)₂D₃ (Fig. 4B), resulting in higher expression of renal Cyp24a1 mRNA. Visually, a correlation could be identified between renal VDR and Cyp24a1 mRNA levels (Fig. 4B), although no correlation was noted between ileal Cyp24a1 mRNA and VDR due to the small change in VDR (Fig. 3B).

The induction pattern of renal Mdr1 mRNA (Fig. 5B), another VDR target gene (44), was similar to that of renal VDR mRNA (Fig. 4A). Induction of both Mdr1 mRNA and P-gp protein was sustained in the kidney after consecutive injections of 1,25(OH)₂D₃ (Fig. 5, B and C). A higher P-gp protein expression (2.7-fold increase) was observed previously for the enhanced renal but not intestinal excretion of digoxin, a P-gp substrate, when the same 1,25(OH)₂D₃ doses were administered to the mouse (11), as found in the present study (Fig. 5C). Thus, VDR regulates Mdr1 and P-gp induction in a tissuespecific manner.

Plasma calcium levels (Fig. 8D) were greatly influenced by temporal changes in mRNA and protein expression of Trpv6, which was increased by 1,25(OH)₂D₃ treatment in both the intestine and kidney (Figs. 3C, 4C, and 8C). The calcium channel Trpv6 together with Trpv5 mediate the transcellular calcium transport following binding to calbindin to facilitate calcium diffusion across the basolateral membrane and extrusion via the ATP-dependent Ca²⁺-ATPase, PMCA1b, and Na⁺/Ca²⁺ exchanger NCX1 (28). Trpv6 is a major contributor for the apical, intestinal absorption of calcium, since a lack of Trpv6 in knockout mice resulted in significant reduction in calcium absorption and plasma calcium levels (5, 16). Calcium balance is intimately related to Trpv5/6, whose channel activities are stimulated by the VDR primarily via the genomic transcription of the VDREs and by the estrogen receptor, ERα (28). The increase in Trpv6 mRNA and protein expression in the mouse intestine and kidney strongly correlates with the increase (10–40%) in plasma calcium (Figs. 3C, 4C, and 8C).
and D) that in turn attenuated plasma PTH level (Fig. 9B) under our dosing regimen of 1,25(OH)2D3. Elevated plasma calcium is expected to activate the CaSR in the parathyroid gland to inhibit synthesis of PTH, which in turn reduces the mRNA expression of renal Cyp27b1 (23, 30). These immediate changes were found in response to the single and multiple 1,25(OH)2D3 doses in our study (Fig. 4D). Changes in ileal and renal Trpv6 protein expression and calcium (Fig. 8, C and D) indirectly regulated renal Cyp27b1 (Fig. 4D) via reduction of PTH (Fig. 9). Moreover, the higher Trpv6 protein content in the duodenum compared with that in kidney (Fig. 8B) suggests that the intestine is the more important organ than the kidney with respect to Trpv6 induction and calcium absorption (Fig. 8D).

Treatment of 1,25(OH)2D3 is known to result in both genomic and nongenomic effects (43), as noted for Cyp24 (25, 40). However, nongenomic effects are difficult to monitor, since these effects occur rapidly (from seconds to a few minutes), exemplified by the opening of calcium channels for calcium influx without changes in gene or protein (43). We believe that chronic treatment of 1,25(OH)2D3 will result in VDR effects that are mostly genomic, since there are notable changes in mRNA and protein levels of VDR target genes, namely, VDR, Cyp24a1, Trpv6, and Mdr1/PGP (Figs. 3–8). Then we examined the correlation between 1,25(OH)2D3 levels vs. mRNA expression of VDR target genes. Our data revealed that the concentrations of 1,25(OH)2D3 in plasma and tissues peaked at 0.5 to 1 h (Fig. 2) and decayed rapidly with a t1/2 of ~6 h (Table 2), and induction of mRNA expression of VDR target genes in the ileum and kidney peaked at 3–9 h (Figs. 3 and 4), but there was no distinct pattern for protein expressions for many of the VDR target genes (Figs. 6–8). In sum, the derived correlation was not always meaningful. A positive correlation would have been expected between the 1,25(OH)2D3 and calcium levels in plasma if nongenomic effects had prevailed; however, a negative correlation was observed (data not shown). As expected, the correlation failed to divulge information on VDR mechanisms. The transduction processes are a multistep phenomenon that may involve multiple organs and multiple feedback or feed-forward loops, which is quite complex, especially with respect to calcium elevation.

Data from this study conclusively show that 1,25(OH)2D3 enters tissues rapidly, shown by the parallel disposition profiles. The terminal half-life and the area under the curve (AUC0–∞) of total 1,25(OH)2D3 (administered + endogenous) remained relatively unchanged between doses, and no trend was identifiable (Table 2 and Fig. 2). The lack of pharmacokinetic changes in drug exposure in these mouse studies is attributed to the immediate, maximal changes in the degradation and synthetic enzymes, Cyp24a1 and Cyp27b1, by VDR activation after the first administration of 1,25(OH)2D3. Hence, repeated dosing renders similar pharmacokinetic effects. A pattern may be discerned for the temporal changes in tissue where concentrations of 1,25(OH)2D3 are correlated to temporal changes in the expression of some VDR target genes. VDR activation increased Trpv6 expression, which was higher in intestine and less so in kidney. Trpv6 is also responsible for the sustained elevation of calcium and attenuation of PTH in plasma upon repeated 1,25(OH)2D3 dosing. With the higher, prevailing calcium concentration, decreased plasma PTH level and Cyp27b1 expression in kidney ensued, rendering a lower synthesis of 1,25(OH)2D3. Rapid induction of Cyp24a1 mRNA and protein expression in kidney hastens 1,25(OH)2D3 clearance, evidenced by plasma 1,25(OH)2D3 falling below basal levels 24 h after single and chronic 1,25(OH)2D3 dosing. The temporal relationships between VDR target genes and 1,25(OH)2D3 levels in tissues and the dose- and route-dependency are currently under investigation with a mechanism-based pharmacokinetic/pharmacodynamic model.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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