Characterization of intestinal phosphate absorption using a novel in vivo method

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With as little as 11.3 ng of 1,25-dihydroxyvitamin D3 per day, at 2 M, suggesting that 1,25-dihydroxyvitamin D3 stimulates active phosphate homeostasis. In intestine, 1,25(OH)2D3 is known to increase intestinal phosphate absorption. The response is saturated as little as 11.3 ng of 1,25-dihydroxyvitamin D3 per day, consistent with a genomic mechanism. The effect of 1,25-dihydroxyvitamin D3 disappears when the dosing solution of phosphate is at 2 M, suggesting that 1,25-dihydroxyvitamin D3 stimulates active transport of phosphate but not diffusion of phosphate. Finally, unlike findings resulting from in vitro or ex vivo experiments, no evidence in vivo was obtained that phosphate absorption requires sodium or is inhibited by potassium.

vitamin D; phosphorus; intestine

THE ACTIVE FORM OF VITAMIN D3, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] is an integral regulator of calcium and phosphate homeostasis. In intestine, 1,25(OH)2D3 is known to stimulate calcium and phosphate absorption (7). Whereas the effect of 1,25(OH)2D3 on intestinal calcium absorption has been extensively studied, the effect of 1,25(OH)2D3 on intestinal phosphate absorption is poorly understood.

Previously, the role of 1,25(OH)2D3 on intestinal phosphate absorption has been studied ex vivo, using everted intestinal segments or Ussing techniques, and in vitro, using brush border membrane vesicles isolated from intestinal tissue. Using these methods, several research groups have demonstrated that 1,25(OH)2D3 increases intestinal phosphate absorption in chicks (14, 19) and rodents (1, 2, 4, 8, 10, 20, 21). Early studies showed that everted intestine or intestinal segments in Ussing chambers require sodium for phosphate transport (3, 11) and that this is inhibited by excess potassium (3). More recent work suggests that this 1,25(OH)2D3-induced increase in intestinal phosphate absorption is mediated by a sodium-dependent phosphate cotransport protein type IIb, Na-PtIib (4, 8, 21). However, the sodium dependence of phosphate absorption has not been demonstrated in vivo.

Previous studies using everted intestinal segments or Ussing techniques followed intestinal phosphate absorption only in a small segment of the intestine ex vivo. However, the ability of 1,25(OH)2D3 to increase phosphate absorption can vary greatly depending on the region of the intestine (10, 13, 20). Furthermore, the rate of phosphate absorption in one segment of the intestine does not necessarily represent the overall rate of phosphate absorption along the entire length of the digestive tract in vivo. Even more troublesome is the use of brush border vesicles as representing transcellular transport. There appears to be a need for measurements of 1,25(OH)2D3-induced intestinal phosphate absorption in a live animal. In this study, we have developed a novel in vivo method for measuring intestinal phosphate absorption by using a radioisotope in rats based on a method previously described for measuring calcium absorption (12).

With this method, we can clearly demonstrate a dose-dependent increase in phosphate absorption in vivo in response to 1,25(OH)2D3. This response is rapid and occurs at low concentrations of phosphate where active transport is dominant and is absent at high concentrations where diffusion is dominant. This method does not confirm a dependence of phosphate absorption on sodium ions or an inhibition by potassium ions in clear contrast to previous results obtained with the ex vivo methods.

MATERIALS AND METHODS

Animals. Male weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) were fed a purified vitamin D-deficient diet (18) for 8 wk ad libitum. Depletion of vitamin D stores was accelerated by alternating an adequate calcium diet with a calcium-deficient diet. Thus, during weeks 1, 5, and 8, the diet contained 0.47% calcium and 0.30% phosphorus. During weeks 2, 3, 4, 6, and 7, the diet contained 0.02% calcium and 0.30% phosphorus. All diets were supplemented with 100 μl of soybean oil (Wesson oil; ConAgra Foods, Irvine, CA) containing 500 μg of α-tocopherol, 60 μg of menadione, and 40 μg of β-carotene three times each week. Rats were housed in hanging wire cages under a UV-filtered 12:12-h light-dark cycle and had free access to distilled water. All experimental methods were approved by the Research Animal Resources Center at the University of Wisconsin-Madison.

1,25-dihydroxyvitamin D3. 1,25(OH)2D3 was purchased from Tetronics/Sigma-Aldrich (Madison, WI) and dissolved in 100% ethanol. The concentration of 1,25(OH)2D3 was calculated according to Beer’s law, using the UV absorbance at 264 nm and an extinction coefficient of 18,200 M−1·cm−1. 1,25(OH)2D3 was then dissolved in vehicle (5% ethanol, 95% propylene glycol). Vehicle (0.1 ml) containing as much as 180 ng of 1,25(OH)2D3 was injected intraperitoneally each day for up to 5 days.

Serum analysis. When rats were injected daily for 5 days, blood was collected from the tail artery under ether anesthesia before the first and fifth injections for serum analysis. When rats were injected only once, blood was collected before the injection in the same manner. Blood was centrifuged at 1,500 g for 15 min at 22°C to yield serum. The serum calcium level was determined by flame atomic absorption spectroscopy.

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absorption spectroscopy (model 3110; Perkin Elmer, Norwalk, CT) using serum diluted 1:40 with 1 g/l LaCl₃. The phosphorus level of untreated serum was determined using a colorimetric method (6).

**Intestinal phosphate absorption.** Following an overnight fast, rats were administered 3 μCi ³²P (as H₃PO₄, specific activity 155.8 Ci/mg; New England Nuclear/PerkinElmer, Boston, MA) in 0.5 ml of a buffer containing 0.25–2,000 mM KH₂PO₄ or NaH₂PO₄ at pH 7.4 via gastric gavage. Unless stated otherwise, rats were administered ³²P ~24 h after the final injection of vehicle or 1,25(OH)₂D₃. Rats were killed by CO₂ asphyxiation immediately or up to 180 min after the oral dose was administered. The rats killed immediately ("0 min control") were used to determine whether the oral ³²P dose was properly administered and completely recovered. Blood was collected via heart puncture and centrifuged as described above to yield serum. A suture was tied to the superior end of the esophagus to contain liquid inside the stomach. The entire digestive tract was then removed and allowed to dissolve for several days in concentrated HNO₃ (~1 ml HNO₃/g tissue). The exact volume of the dissolved digestive tract was determined following liquid scintillation counting of 50-μl aliquots in triplicate (Tri-Carb liquid scintillation analyzer; PerkinElmer/Packard, Boston, MA). Total body serum was estimated to be 40 ml serum/kg body wt (5).

**Statistical analysis.** Data are presented as means ± SE. Treatment groups were compared using a fully factorial analysis of variance, and means were subjected to Tukey, Scheffé, and Fisher’s least significant difference tests (Systat 5.2.1; Systat Software, Point Richmond, CA). Differences were considered significant if at least two of the tests detected significance with a P value < 0.05.

**RESULTS**

**Time course of intestinal phosphate absorption.** Intestinal phosphate absorption was measured 30, 45, 60, 90, and 180 min after administration of an oral ³²P dose in vitamin D-deficient rats injected with vehicle or 90 ng of 1,25(OH)₂D₃ each day for 5 days. Reduced serum calcium levels measured before the injection demonstrated that the rats were vitamin D deficient (Fig. 1A). As expected, serum calcium values significantly increased after the injection of 1,25(OH)₂D₃, but not vehicle. Serum phosphorus levels significantly increased in both vehicle- and 1,25(OH)₂D₃-injected rats (Fig. 1B). Intestinal phosphate absorption from 1,25(OH)₂D₃-treated rats was significantly higher than vehicle-treated rats 30, 45, and 60 min after administration of an oral ³²P dose (Fig. 1C). Accordingly, a significant increase in serum ³²P was detected at 30 and 60 min after administration of the oral ³²P dose (Fig. 1D).

**Dose response to 1,25(OH)₂D₃.** Intestinal phosphate absorption was measured 60 min after administration of an oral ³²P dose in vitamin D-deficient rats injected with vehicle or as much as 180 ng of 1,25(OH)₂D₃ each day for 5 days. Serum calcium values were significantly increased when 11.3–180 ng of 1,25(OH)₂D₃ were injected (Fig. 2A). Serum phosphorus levels were increased in all groups but only reached statistical significance with the serum phosphorus levels measured in rats injected with 11.3 or 180 ng of 1,25(OH)₂D₃. An increase in intestinal ³²P absorption was also detected in rats dosed with 11.3–180 ng of 1,25(OH)₂D₃ (Fig. 2C). Although serum ³²P levels were increased in rats dosed with all levels of 1,25(OH)₂D₃ (Fig. 2D), only the levels from the group injected with 90 ng of 1,25(OH)₂D₃ reached statistical significance.

**Response to a single dose of 1,25(OH)₂D₃.** Rats were injected with a single dose of vehicle or 135 ng of 1,25(OH)₂D₃, followed by an oral dose of radiolabeled phosphate 0, 8, or 24 h after the injection. Rats were then killed 60 min after administration of the oral ³²P dose. Serum calcium levels were measured from blood samples collected via tail artery before the injection and from the blood collected via heart puncture at death. A significant increase in serum calcium levels was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A). A significant increase in intestinal ³²P absorption was observed 24 h after the 1,25(OH)₂D₃ injection (Fig. 3A).

**Effect of phosphate concentration on 1,25(OH)₂D₃-stimulated phosphate absorption.** Increasing the concentration of phosphate from 0.25 to 2,000 mM in the oral dose eliminated...
the effect of 1,25(OH)2D3 (Fig. 4A). Increases in serum calcium and phosphorus levels similar to those shown in Fig. 1, A and B, were observed (data not shown). There were significant increases in intestinal 33P absorption (Fig. 4A) and serum 33P levels (Fig. 4B) in 1,25(OH)2D3-treated rats administered 33P in 0.25 to 1,000 mM unlabeled phosphate. However, when 33P was administered in 2,000 mM phosphate, there was no difference in intestinal 33P absorption or serum 33P levels between vehicle- and 1,25(OH)2D3-treated rats.

Does intestinal phosphate absorption require sodium or potassium? Intestinal 33P absorption was measured 60 min after administration of an oral dose of 33P in 0.5 mM KH2PO4 or NaH2PO4 in vitamin D-deficient rats injected with vehicle or 90 ng of 1,25(OH)2D3. Serum calcium and phosphorus levels were increased in a manner similar to that shown in Fig. 1, A and B (data not shown). There was a significant increase in intestinal 33P absorption (Fig. 5A) and absorption of 33P into serum (Fig. 5B) in rats injected with 1,25(OH)2D3 when 33P was administered in a buffer containing either form of phosphate.
DISCUSSION

To study the in vivo absorption of phosphate, we have devised a new method based on a method previously developed for studying intestinal calcium absorption. This method relies on the intragastric administration of inorganic phosphate and a measurement of disappearance of that phosphate from the gastrointestinal (GI) tract over a period of time. An ancillary measurement is the appearance of radioactive phosphate in the plasma. To minimize dilution of the intragastrically administered radiolabeled phosphate with unlabeled dietary phosphate, the animals were fasted for 16 h. At various times following gastric administration of the $^{33}$P-labeled phosphate, the entire GI tract was removed and digested to measure the amount of phosphate remaining in the GI tract. The amount that disappeared was considered phosphate absorbed during that period. A period of 60 min of absorption was determined as optimum, because at longer periods most of the phosphate disappears from the intestinal tract, and hence, the rate of phosphate absorption could no longer be measured.

Another important facet of this method is that the phosphate was administered at a concentration of ~0.5 mM. This concentration approaches the concentration found in the plasma, and hence, disappearance from the GI tract into the plasma compartment would require cellular and metabolic participation. With the use of this method and measurement of both the disappearance of $^{33}$P from the intestine and the appearance of $^{33}$P in the plasma, it is clear that the administration of the active form of vitamin D, 1,25(OH)$_2$D$_3$, significantly stimulates the rate of phosphate absorption in vivo. This conclusion was clearly deduced earlier by investigators using a variety of ex vivo measurements including Ussing chamber methods as well as everted sacs. It is of considerable interest that phosphate is quite rapidly absorbed even in the absence of 1,25(OH)$_2$D$_3$. When the phosphate concentration of the administered dose was increased progressively to 2,000 mM, the effect of 1,25(OH)$_2$D$_3$ on the rate of phosphate disappearance from the GI tract was eliminated. With these concentrations of phosphate in the GI tract, the primary mechanism of absorption is presumably diffusion, which may or may not involve cellular participation. Our results are consistent with the idea that 1,25(OH)$_2$D$_3$ does not affect the absorption of phosphate by the mechanism operating at 2,000 mM phosphate. These results, therefore, are consistent with the idea that vitamin D stimulates active phosphate absorption, presumably through a transcellular mechanism.

Fig. 4. Competition with unlabeled phosphorus. Vitamin D-deficient rats were injected intraperitoneally each day with vehicle or 90 ng of 1,25(OH)$_2$D$_3$ for 5 days. Rats were fasted overnight, dosed orally with 3 μCi $^{33}$P in a buffer containing 0.25 mM to 2,000 mM KH$_2$PO$_4$, and killed after 60 min. A: percentage of oral $^{33}$P dose remaining in digestive tract. *P < 0.05, significantly different from vehicle-treated rats administered the same KH$_2$PO$_4$ concentration. B: percentage of oral $^{33}$P dose detected in serum. *P < 0.05, significantly different from vehicle-treated rats administered the same KH$_2$PO$_4$ concentration.

Fig. 5. Does phosphate absorption require sodium or potassium? Vitamin D-deficient rats were injected intraperitoneally each day with vehicle or 90 ng of 1,25(OH)$_2$D$_3$ for 5 days. Rats were fasted overnight, dosed orally with 3 μCi $^{33}$P in a buffer containing 0.5 mM Na$_2$HPO$_4$ or KH$_2$PO$_4$, and killed after 60 min. A: percentage of oral $^{33}$P dose remaining in digestive tract. *P < 0.05, significantly different from vehicle-treated rats administered the same form of phosphate. B: percentage of oral $^{33}$P dose detected in serum. *P < 0.05, significantly different from vehicle-treated rats administered the same form of phosphate.
The mechanism whereby 1,25(OH)\(_2\)D\(_3\) stimulates intestinal phosphate absorption is easily saturated by the administration of increasing amounts of 1,25(OH)\(_2\)D\(_3\). This is consistent with a mechanism involving the vitamin D hormone interacting with its receptor in a genomic action, causing the expression of genes presumably including a phosphate transporter, at a minimum.

Because of the previously carried out in vitro experiments, we had suspected that phosphate absorption in vivo might be dependent on sodium and might not occur when the phosphate is presented as the potassium salt. We found no difference in the ability of the intestine to absorb phosphate whether it was presented as the sodium or potassium salt. Although it is possible that cellular sodium exchanged for the potassium and, therefore, allowed the sodium-dependent phosphate transport to occur, we were surprised that we were unable to provide evidence that sodium stimulates whereas potassium inhibits phosphate absorption.

Our results using this in vivo method of measuring phosphate absorption support very strongly the suggestion that phosphate absorption is, in fact, stimulated by the active form of vitamin D in a saturable process, very likely by effecting an active, cellulary mediated process. Our results do not support the idea that 1,25(OH)\(_2\)D\(_3\) affects the diffusional mechanism of absorbing phosphate, and furthermore, we found no support for the idea that phosphate absorption is a sodium-dependent process or that it is inhibited by potassium.

Previous work using ex vivo and in vitro systems has suggested that intestinal phosphate absorption is stimulated by low dietary calcium or low dietary phosphorus, yet these findings remain to be tested in a whole animal system (4, 8, 9, 13–15). More recently, an additional factor, fibroblast growth factor-23 (FGF23) has been shown to be important for maintaining phosphate homeostasis by decreasing renal phosphate reabsorption while suppressing the synthesis of 1,25-(OH)\(_2\) vitamin D. This results in hyperphosphatemia and impaired skeletogenesis, and reverses hypophosphatemia in Phex-deficient mice. Therefore, the idea that phosphate absorption is a sodium-dependent process or that it is inhibited by potassium.

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