NON RUMINANT NUTRITION

The effect of dietary vitamin D supplementation on sodium-dependent phosphate uptake and expression of NaPi-IIb in the small intestine of weanling pigs

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Abstract

Two experiments were conducted to investigate the effects of 1,25(OH)₂D₃ to stimulate Na⁺-dependent phosphate uptake in Caco-2 cells, and the effects of dietary vitamin D supplementation to vitamin D-deficient nursery pigs on Na⁺-dependent nutrient uptake and mRNA expression of NaPi-IIb cotransporter and calbindin D9k in the jejunum. In Exp. 1, 250,000 Caco-2 cells were seeded on Costar 12 mm Snapwell inserts with a 0.40 μm polycarbonate filter and a seeding density of 0.25 × 10⁶ and studied at 15 d postconfluence. Cells were treated with 10 nM of either 1,25(OH)₂D₃ or vehicle for 48 h and then mounted in modified Ussing chambers for transepithelial measurements. In Exp. 2, pigs (*n* = 32) were removed from sows at 3 d of age, placed on a vitamin D-deficient milk replacer diet and housed in a room devoid of sunlight and UV light in the range of 280 to 300 nm. On day 28, serum 25(OH)D₃ concentrations were measured to verify low vitamin D status. Pigs (BW 10.10 ± 0.38 kg) were then individually housed day 28 postweaning and allotted to 1 of 2 dietary treatments. Dietary treatments consisted of corn-soybean-based diets with vitamin D supplementations of 0 or 1,500 IU/kg diet for 12 d. Blood samples were taken from the brachiocephalic vein on the initial (day 0) and final day (day 10, 11, or 12) of the study for analysis of serum 25(OH)D₃, P, and Ca. Pigs were euthanized and jejunal segments were harvested and used in modified Ussing chambers and for RNA isolation and subsequent quantitative RT-PCR analysis. In Exp. 1, treating Caco-2 cells with 10 nM 1,25(OH)₂D₃ resulted in a 52% increase (*P* < 0.005) in Na⁺-dependent phosphate uptake compared with cells treated with a vehicle. In Exp. 2, Na⁺-dependent phosphate and glucose transport did not differ (*P* > 0.10) among treatment groups. Additionally, NaPi-IIb and calbindin D9k mRNA expression were not different (*P* > 0.10) between treatment groups. No differences (*P* > 0.10) were detected in final serum P or 25(OH)D₃ concentrations between treatments. However, serum Ca linearly increased (*P* < 0.05) as the concentration of supplemental vitamin D increased in the diet. Overall, while 1,25(OH)₂D₃ stimulated Na⁺-dependent phosphate uptake in Caco-2 cells, supplementing diets with 1,500 IU/kg vitamin D₃ from cholecalciferol did not increase jejunal Na⁺-dependent phosphate uptake or NaPi-IIb mRNA expression over that of pigs fed diets with no supplemental cholecalciferol.

Key words: phosphate, small intestine, swine, vitamin D

Introduction

Environmental pressures have increased in the past decade to limit P buildup in both soil and waterway and emphasis has been placed on limiting P excretion in animal manure (Mann and Grant, 2015). Additionally, costs of rock phosphates have increased in the past few years and are not expected to decline. To prevent increases in diet costs, alternatives are being sought to replace inorganic rock phosphates in the diet. Developing nutritional methods to improve P absorption in the small intestine could allow for less inorganic rock phosphates to be included in the diet and decrease P excretion in the manure. However, a lack of understanding of mechanisms involved in P absorption in the small intestine has limited research focused on improving absorption efficiency of P in the small intestine. The efficiency of intestinal phosphate absorption is greatest when dietary concentrations of P are limiting and declines as the requirement is met (Anderson, 1991). Increases in absorption efficiency, under conditions when P intake is limiting, is due to an increase in Na⁺-dependent phosphate uptake in the intestine (Hattenhaur et al., 1999). Intravenous administration of 1,25(OH)₂D₃ has been shown to stimulate Na⁺-dependent uptake in the small intestine of rats (Katai et al., 1999). Furthermore, direct feeding of cholecalciferol increases P retention in chicks fed low-P diets (Edwards, 1993). However, it is still unclear whether 1,25(OH)₂D₃ or cholecalciferol simulate active P uptake in the small intestine of pigs. The objective of Exp. 1 was to determine if 1,25(OH)₂D₃ could stimulating active P uptake in a Caco-2 cell model. The objective of Exp. 2 was to determine if feeding cholecalciferol in excess of the requirement stimulated Na⁺-dependent phosphate uptake in the small intestine of weanling pigs with low vitamin D status.

Materials and Methods

Cell Culture, Exp. 1

A parental Caco-2 cell line (HTB37, ATCC, Rockville, MD) was grown and passaged in 75-cm² flasks when 75% to 80% confluent by dispersion in 0.1% trypsin. Cells were studied between passages 25 and 50. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal bovine serum, 10 mM nonessential amino acids, 200 mM L-glutamine, 100 U/L penicillin, 100 µg/L streptomycin mixture, 100 mM sodium pyruvate, 50 mg/mL gentamycin sulfate, and 10 mM HEPES. Cells were incubated at 37 °C, in a 5% CO₂-95% air environment. For transepithelial P transport studies, ~250,000 Caco-2 cells were seeded onto Costar 12 mm Snapwell inserts (Corning, Inc., Corning, NY) with a 0.40-µm polycarbonate filter. On day 15 postconfluency, cells were treated with either 10 nM 1,25(OH)₂D₃ (n = 8) or vehicle (ethanol, 0.01%, n = 6) for 48 h. Treatments were diluted in DMEM + 5% fetal bovine serum.

Animals and Treatments, Exp. 2

All animal procedures were approved by Purdue's Animal Care and Use Committee. Thirty-six crossbred gilts (2.342 ± 0.37) were used in 2 replicate trials (n = 18/replicate). Pigs were weaned 72 h after birth from sows fed adequate vitamin D in standard commercial farrowing rooms and placed in a room devoid of both sunlight and UV light in the range of 280 to 300 nm to prevent formation of 7-dehydroxycholesterol in the skin by exposure to UV light. Pigs were group housed in pens (1.2 m²) and placed on a milk replacer (Table 1) devoid of vitamin D supplementation but formulated to meet or exceed all other

Table 1. Composition of milk replacer diet

Vitamin D supplementation, IU/kg diet	0
Ingredients	---%---
Whey powder	20.0
Whey protein concentrate	21.7
Lactose	10.0
MSC fat (12/28) ¹	2.25
MSC fat (7/60) ²	28.3
Swine vitamin premix ³	2.514
Vegetarian component ⁴	15.25
Calculated composition	
ME, kcal/kg	
Phosphorus, %	0.66
Calcium, %	0.50
Lysine	2.00
Crude protein	26.6
Analyzed composition	
Phosphorus, %	0.78
Calcium, %	1.13
Dry matter, %	89.4
Vitamin D, IU/kg	ND

¹Contained animal fat, condensed whey, dried protein concentrate, mono and diglycerides of vegetable oil, and sodium silico aluminate. Product contained 12% CP and 28% fat (MSC Specialty Nutrition).

²Contained animal fat, condensed whey, dried protein concentrate, mono and diglycerides of vegetable oil, and sodium silico aluminate. Product contained 7% CP and 60% fat (MSC Specialty Nutrition).

³Vitamin premix provided the following guaranteed minimums per kg diet: vitamin A, 9,000 IU; vitamin E, 187 IU; vitamin K (hetrazeen), 2.62 mg; vitamin B₁, 1.857 mg; vitamin B₁₂, 17.25 µg; riboflavin, 5.25 mg; d-pantothenic acid, 11.25 mg; niacin, 18.75 mg.

⁴Contained a mixture of wheat gluten, soy protein isolate, and soy protein concentrate.

nutrient requirements (NRC, 1998). Pigs were fed the liquid milk replacer ad libitum for 28 d. Pigs were fed with a recirculating milk feeding system. The system was cleaned daily and fresh milk was provided daily. Blood samples were taken on day -28, -21, -14, -7, 0, and 10 or 12. Vitamin D status was evaluated by serum 25(OH)D₃ concentrations (25(OH)D₃ kit, IDS, Fountain Hills, AZ) and pigs were considered deficient when values were less than 25 nmol/mL (Hart, 2004, 2005). Additionally, serum Ca and P concentrations were measured at all time points. On day 28, piglets were blocked by body weight and randomly assigned to 1 of 2 dietary treatments and housed in individual pens (1.2 m²). Dietary treatments were corn-soybean meal based and contained 0 (n = 24) or 1,500 IU/kg (n = 12) of supplemental vitamin D (Table 2). Diets were formulated to meet or exceed all NRC (1998) requirements except for vitamin D. Pigs were allowed ad libitum access to both feed and water.

Intestinal Segments

On day 10, 11, or 12 following initiation of dietary treatments, pigs were euthanized by CO₂ asphyxiation at a common final BW, with the heaviest block being euthanized on day 10 and the lightest on day 12. Intestinal segments were collected 200 cm posterior from the pyloric junction for transepithelial flux rate measurements in modified Ussing chambers (Physiologic Instruments, Inc., San Diego, CA). Additionally, mucosal scrapings

Table 2. Composition of dietary treatments

Vitamin D supplementation, IU/kg diet	0	1,500
Ingredients	-----%-----	
Corn	47.7	47.7
Soybean meal, 48% CP	12.4	12.4
Whey	25.0	25.0
Soybean oil	4.35	4.35
Fish meal	4.00	4.00
Plasma protein	5.00	5.00
Calcium carbonate	0.87	0.87
Salt	0.20	0.20
Swine trace mineral mix ¹	0.13	0.13
Swine vitamin premix ²	0.10	0.10
L-Lysine-HCl	0.10	0.10
D,L-Methionine	0.05	0.05
Se600 ³	0.05	0.05
Sulkaflor	0.05	-
Vitamin D ⁴		0.05
Calculated composition		
ME, kcal/kg	3267	3267
Phosphorus, %	0.60	0.60
Calcium, %	1.00	1.00
Lysine	1.35	1.35
Analyzed composition		
Phosphorus, %	0.70	0.71
Calcium, %	1.21	1.22
Dry matter, %	93.0	93.0
Vitamin D, IU/kg	348	1125

¹Trace mineral premix provided the following guaranteed minimums per kg diet: iron, 84.7 mg; zinc, 84.7 mg; manganese, 10.5 mg; copper, 7.87 mg; iodine, 0.32 mg.

²Vitamin premix provided the following guaranteed minimums per kg diet: vitamin A, 9,000 IU; vitamin E, 187 IU; vitamin K (hetrazen), 2.62 mg; vitamin B₁, 1.857 mg; vitamin B₁₂, 17.25 µg; riboflavin, 5.25 mg; d-pantothenic acid, 11.25 mg; niacin, 18.75 mg.

³Premix provided 0.3 mg Se per kg of the diet.

⁴Cholecalciferol, contained 3,000 IU/g.

were collected for RNA isolation and preparation of brush border membrane vesicles approximately 200 cm posterior from the pyloric junction. Segments taken for the Ussing chambers were immediately placed in a modified P-free Krebs buffer solution (124 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 26 mM NaHCO₃, and 5 mM glucose, pH 7.4) and continuously aerated at 4 °C until mounting in the Ussing chambers. Jejunal sections were also scraped with a glass slide and ~2.0 g mucosa was flash-frozen with liquid nitrogen for RNA isolation.

Ussing Chambers

Techniques assessing Caco-2 cells and gastrointestinal function through ion flux measurements in modified Ussing chambers have been previously described (Castro et al., 1987; Kles et al., 2001; Schaar et al., 2004). The growth media was aspirated from the Caco-2 cells and cells were then washed 3 times in prewarmed (37 °C) modified phosphate-free Krebs buffer (124 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 26 mM NaHCO₃, and 5 mM glucose). This allowed for the removal of any residual media and allowed monolayer to adapt to the new buffer. Jejunal segments were stripped of the outer serosal layer and mounted in modified Ussing chambers equipped to measure transmural short-circuit current, and resistance. Tissues were

mounted in pairs and the area of tissue exposed was 1.00 cm². Tissues were bathed in 8 mL of modified phosphate-free Krebs buffer. Both Caco-2 cells and tissues were maintained at 37 °C with a circulating water bath (VWR, Batavia, IL) and oxygenated in 95% O₂/5% CO₂. Basal transepithelial short-circuit current (*I*_{sc}) and resistance (TER) were measured after a 15-min equilibration period. Transepithelial resistance was calculated from Ohms law by the computer software (Acquire and Analyze software, Physiologic Instruments) which was determined from the magnitude of the current deflections in response to a voltage pulse (0.1 mV) imposed on the short-circuited cells/tissue every 5 s with a duration of 0.5 s. Only Caco-2 cell monolayers generating a TER of at least 150 ohm/cm² (indicating a mature monolayer) were used in transport studies. Sodium-dependent nutrient transport was determined by measuring changes in short-circuit current induced by the addition of 10 mM inorganic phosphate (Na₂HPO₄) or glucose to the mucosal side which was osmotically balanced on the serosal side by addition of 10 mM mannitol. Chloride secretion was induced by the addition of 10 mM carbachol and serotonin to the serosal side and was osmotically balanced by the addition of 10 mM mannitol to the mucosal side of the tissue to verify tissue viability at the end of the experiment. The modified Ussing chambers were connected to dual channel voltage/current clamps (VCC MC2; Physiologic Instruments, San Diego, CA) with a computer interface allowing for real-time data acquisition and analysis (Acquire and Analyze software, Physiologic Instruments).

Real-Time PCR

Total RNA was isolated from jejunal mucosa using Tri-Reagent per the manufacturer's directions (Molecular Research Center, Cincinnati, OH). Total RNA (1.0 µg) was reverse transcribed into first-strand cDNA with an oligo-dT primer using M-MLV transcriptase (Invitrogen) as previously described (Fleet and Wood, 1999). Real-time PCR was conducted on samples using the BioRad My iQ RT-PCR system containing SYBR green (BioRad). NaPi-IIb, calbindin D9k, and GAPDH mRNA concentrations were determined from the threshold cycle value (Livak and Schmittgen, 2001) and were normalized to the expression of GAPDH within the sample. These values were then standardized such that a value of 1.0 was assigned to the control group. Primers and annealing temperatures were: NaPi-IIb, forward: 5'-CTCTGTAGCTGCCTGGTCTCTAA-3', reverse: 5'-GGT CAGAGTCGACGAGAACAC-3', annealing temperature (*T*_a) = 53 °C; calbindin D9k, forward: 5'-CAAACCAGCTGTCTGAAGGA-3', reverse: 5'-TAGGGTTCTCGGACCTTTTCAG-3', *T*_a = 54 °C; GAPDH, forward: 5'-TCACCATCTTCCAGGAGCG-3', reverse: 5'-CTGCTTCACC ACCTTCTTGA-3', *T*_a = 54 °C.

The PCR reactions were conducted for 42, 35, 35, and 32 cycles for NaPi-IIb, calbindin D9k, and GAPDH, respectively, to ensure amplification efficiency in the linear range of each primer set. Product identities were confirmed by melting curve analysis (Livak and Schmittgen, 2001).

Chemical Analysis

Diet samples were ground to pass through a 1 mm screen. Dry matter content of diets was determined by drying at 105 °C to a constant weight. Diet samples were digested using a 1:3 ratio (vol/vol) of 60% perchloric acid and 70% nitric acid in tubes at 100 °C for 1.5 h and 200 °C for 3.5 h. The P content was determined colorimetrically using a molybdo vanadate assay (AOAC method 965.17, 1990) using a microplate reader (550 Microplate Reader, BioRad, CA). Serum P concentrations were determined by the method of Chen et al. (1956) and the procedure was modified

with AOAC (method 965.17, 1990). Calcium concentration was determined with an atomic absorption spectrophotometer (Spectra AA 220Z, Varian, Australia). Diets were also assayed for vitamin D (DSM Nutritional Products, Pomfret Center, CT).

Statistical Analysis

The experiment was a completely randomized block design. The cholecalciferol supplementation data were analyzed for main effects of treatment, block, and treatment $\times$ block using the GLM procedure in SAS (SAS Inst. Inc., Cary, NC). Serum Ca, P, and $25(\text{OH})_2\text{D}_3$ were analyzed with repeated measures. Data were analyzed with pig as the experimental unit. Duncan's multiple range test was utilized if treatment means differed by $P < 0.05$. Differences were considered significant at $P < 0.05$ and trends at $P < 0.10$. Normality was assessed using the proc univariate procedure in SAS and homogeneity of the variance using residual plots. To achieve normality, day 28 pig body weights and ADG were transformed using the y^2 function (SAS Inst. Inc., Cary, NC). Additionally, initial serum Ca, P, and final serum Ca concentrations were transformed using the y^4 function to achieve normality (SAS Inst. Inc., Cary, NC). Finally, initial and final serum $25(\text{OH})_2\text{D}_3$ concentrations were transformed using the $y^{-1.5}$ function to achieve normality (SAS Inst. Inc., Cary, NC). Statistics are reported on the transformed data but reported means and standard errors are from the untransformed data. Values are reported as means $\pm$ SE.

Results and Discussion

Cell Culture

Caco-2 cells are a human colon adenocarcinoma cell line that has been widely used for intestinal transport studies since they can spontaneously differentiate into polarized epithelial cells with functional properties of transporting epithelia of the small intestine (Hidalgo et al., 1989). Caco-2 cells have proven to be an appropriate model to study the transport of nutrients such as amino acids, minerals, and drugs (Hidalgo and Borchardt, 1990; Fleet et al., 2002). Treating Caco-2 cells with 10 nM $1,25(\text{OH})_2\text{D}_3$ for 48 h resulted in a 52% increase ($P < 0.005$) in Na^+ -dependent phosphate transport compared with cells treated with a vehicle (Fig. 1). As expected, no differences ($P > 0.10$) were detected in Na^+ -dependent glucose uptake or chloride secretion (data not shown) in cells treated with either $1,25(\text{OH})_2\text{D}_3$ or vehicle. However, all cells were capable of transporting glucose and

Figure 1. The effect of 10 nM $1,25(\text{OH})_2\text{D}_3$ or vehicle on active ion transport in Caco-2 cells. Cells were challenged in modified Ussing chambers with 10 mM phosphate on the mucosal side and osmotically balanced with 10 mM mannitol on the serosal side. Baseline I_{sc} values were subtracted from I_{sc} values obtained 15 min post-challenge to calculate the change in I_{sc} . Values are reported as means $\pm$ SE. Treatment effect ($P < 0.005$).

secreting chloride proving cells were viable during the transport studies. These data show that active P transport can be directly stimulated by $1,25(\text{OH})_2\text{D}_3$ in cells possessing characteristics similar to that of the small intestine. Further research will need to be conducted to confirm if the increase in Na^+ -dependent P transport was due to an increase in the NaPi-IIb cotransporter protein and/or mRNA. While Caco-2 cells are a good model for intestinal transport, limitations exist since they are an isolated cell system lacking mucus or the underlying submucosal and muscle tissues. Due to these limitations, the effect of directly feeding vitamin D_3 from cholecalciferol was investigated in an animal model.

Pig Vitamin D Status

In attempt to standardize vitamin D status, pigs were depleted of vitamin D prior to consuming diets with 0 or 1,500 IU/kg vitamin D from cholecalciferol. To limit vitamin D consumption, but still receive colostrum from the sows milk, pigs were weaned at 3 d of age ($\text{BW} = 2.34 \pm 0.09$ kg). All pigs were placed in a room devoid of sunlight and UV light in the range of 280 to 300 nm to prevent formation of 7-dehydroxycholesterol in the skin. Dry matter intake was 347 ± 0.50 g/d and ADG was 280 ± 11.6 g/d during the 28-d period. Pigs ended the 28-d period with a final BW of 10.20 ± 0.39 kg (Table 3). Several authors have reported that supplementing suckling pigs with a liquid milk replacer results in increased growth rate and weaning weight (Azain et al., 1996; Wolter et al., 2002). In the current study, ADG of pigs weaned on day 3 post-farrowing, fed only the vitamin D-deficient milk replacer, was greater than values reported for both suckling pigs and suckling pigs supplemented with liquid milk replacer (Wolter et al., 2002).

Blood samples were taken weekly to measure serum $25(\text{OH})_2\text{D}_3$ concentrations to monitor vitamin D status. Initial serum $25(\text{OH})_2\text{D}_3$ concentrations (day 3 age) were 65.58 ± 4.5 nmol/L (data not shown), and by day 28 postweaning, serum concentrations for all pigs were below 25 nmol/L (19.36 ± 4.0 nmol/L) which was the threshold level used to determine vitamin D deficiency (Hart, 2004, 2005). Dietary Ca concentrations were kept adequate to prevent results being confounded by a Ca deficiency and secondary hyperparathyroidism. Pigs ended the 28-d period with serum Ca and P concentrations of 9.81 ± 1.42 mg/dL and 9.41 ± 2.22 mg/dL. Studies with rats have shown vitamin D deficiency can be induced in rats without feeding a classic rachitogenic diet (low Ca and low P) and hence avoid declines in serum Ca and P concentrations, and increases in parathyroid hormone concentration (Lester et al., 1982; Kollenkirchen et al., 1991). Additionally, feeding a vitamin D-deficient diet that is adequate in Ca and P is reported to result in the most rapid decline in serum $25(\text{OH})_2\text{D}_3$, and coincides with a significant decrease of serum $1,25(\text{OH})_2\text{D}_3$. This is in contrast to animals fed a vitamin D-deficient diet with insufficient P and Ca in which the decline in serum $25(\text{OH})_2\text{D}_3$ precedes a significant decline in serum $1,25(\text{OH})_2\text{D}_3$ by 4 wk (Kollenkirchen et al., 1991). Results from our study indicate that while serum $25(\text{OH})_2\text{D}_3$ concentrations were at deficient concentrations (<25 nmol/L), serum Ca and P remained within normal ranges (Mahan, 1982). Our study indicates that if pigs are removed from the sow at 3 d of age, it is possible to induce low $25(\text{OH})_2\text{D}_3$ status within 28 d while still maintaining adequate Ca and P status.

Vitamin D Supplementation

Vitamin D analysis of the dietary treatments revealed diets contained 348 and 1,125 IU/kg. Diets with no supplemental cholecalciferol were more than the NRC (1998) vitamin D

requirement of 200 IU/kg, indicating that the dietary ingredients provided a sufficient amount of vitamin D to meet the requirements of the animal. The addition of vitamin D, either through dietary ingredients or addition of cholecalciferol in diets of pigs with reduced serum 25(OH) $_2$ D $_3$ resulted in an increase in final serum Ca (time effect, $P = 0.016$), serum P (time effect, $P = 0.065$), and 25(OH) $_2$ D $_3$ ($P < 0.001$). However, supplementation of 1,500 IU/kg of vitamin D $_3$ only tended to increase serum Ca (Trt effect, $P = 0.056$) and had no effect on serum P ($P = 0.100$) or serum 25(OH) $_2$ D $_3$ ($P = 0.937$) compared with pigs fed a diet with no vitamin D provided by the addition of cholecalciferol (Fig. 2). Holtrop et al. (1986) reported a significant increase in serum Ca, P, 25(OH) $_2$ D $_3$, and 1,25(OH) $_2$ D $_3$ with the addition of supplemental dietary vitamin D to vitamin D-deficient rats. The increase in 25(OH) $_2$ D $_3$ concentration in both treatment groups indicates all pigs had adequate vitamin D status at the end of the experiment (Hart, 2004, 2005). However, feeding 1,500 IU/kg vitamin D had no effect ($P > 0.10$) on final serum P or 25(OH) $_2$ D $_3$ concentrations compared with pigs fed no supplemental vitamin D $_3$ from cholecalciferol. Avila et al. (1999) reported increasing the dietary vitamin D concentration from 0 to 40,000 IU/kg had no effect on plasma, 25(OH) $_2$ D $_3$, and 1,25(OH) $_2$ D $_3$ concentrations in rainbow

trout, but the authors did report serum P was linearly increased with the addition of increasing concentrations of cholecalciferol. However, this is likely due to the 27-fold greater supplemental dietary vitamin D concentrations fed in comparison to the 5.6-fold greater supplemental dietary vitamin D concentrations in relation to the requirements of the animals fed in the present study.

Na-Dependent Nutrient Uptake and Gene Expression

Our next objective was to determine if dietary cholecalciferol stimulates active phosphate transport in the small intestine. The jejunum was chosen as the intestinal segment since it has been reported to be the only region to respond to i.p. injections of 1,25(OH) $_2$ D $_3$ in both mice and rats (Marks et al., 2006). Initial basal short-circuit current, a measure of total active ion transport, was not different ($P > 0.10$) between pigs fed varying concentrations of supplemental cholecalciferol (data not shown). Additionally, initial resistance, a measure of total passive ion transport, was not different ($P > 0.10$) between treatment groups (data not shown). The change in short-circuit current induced by the addition of phosphate between pigs fed either no supplemental cholecalciferol or 1,500 IU/kg supplemental vitamin D $_3$ from cholecalciferol (Fig. 3) was not different ($P > 0.10$), indicating Na $^+$ -dependent phosphate uptake was similar between treatment groups. However, since no negative control diet (0 IU Vit D/kg diet) was present, it is impossible to determine if vitamin D supplementation stimulates active P transport in the small intestine of pigs with reduced vitamin D status. However, feeding a vitamin D concentration 5.6 times more than the requirement failed to further stimulate active P transport over pigs fed the NRC requirement. These data are in agreement with Avila et al. (1999) which reported feeding graded concentrations of dietary vitamin D (0–40,000) failed to enhance *in vivo* Pi uptake in the intestine of rainbow trout. It is possible that increases in serum P reported by Avila et al. (1999) in response to feeding increasing increments of cholecalciferol were due to an increase in renal P reabsorption rather than increase in intestinal absorption. Additionally, animal studies conducted with both deficient and adequate vitamin D status rats s.c. injected with 1,25(OH) $_2$ D $_3$ report a 2-fold stimulation in phosphate transport in intestinal brush border membrane vesicles (Katai et al., 1999). Increased vascular concentrations of 1,25(OH) $_2$ D $_3$ appear to be necessary for 1,25(OH) $_2$ D $_3$ to stimulate intestinal P transport. Nemere (1996)

Figure 2. The effect of dietary vitamin D supplementation on (A) serum Ca concentrations; time effect, $P = 0.016$, Trt effect, $P = 0.057$, (B) serum P concentrations; time effect, $P = 0.065$, Trt effect, $P = 0.10$, and (C) serum 25(OH) $_2$ D $_3$; time effect, $P < 0.001$, Trt effect, $P = 0.938$ in weanling pigs. Values are reported as means $\pm$ SE.

Figure 3. The effect of dietary vitamin D supplementation on active ion transport in the small intestine of weanling pigs. Tissues were challenged in modified Ussing chambers with 10 mM phosphate and glucose on the mucosal side and osmotically balanced with 10 mM mannitol on the serosal side. Baseline I_{sc} values were subtracted from I_{sc} values obtained 15 min post-challenge to calculate the change in I_{sc} . Values are reported as means $\pm$ SE. Differences were not detected between dietary treatments ($P > 0.10$).

Figure 4. The effect of dietary vitamin D supplementation on gene expression in the small intestine of weanling pigs. Gene expression is reported as amount relative to the housekeeper gene, GAPDH. Values are reported as means $\pm$ SE. Differences were not detected between dietary treatments ($P > 0.10$).

reported that luminal perfusion with 65 pM $1,25(\text{OH})_2\text{D}_3$ in the duodenal loop failed to stimulate phosphate transport greater than that of control chicks while vascular perfusion resulted in a 160% increase in phosphate transport into the venous effluent compared with control chicks. This is logical since both proteins reported to be involved in the ability of $1,25(\text{OH})_2\text{D}_3$ to stimulate P transport, the vitamin D receptor (VDR) and the $1,25(\text{OH})_2\text{D}_3$ -membrane-associated rapid response steroid (MARRS)-binding protein are both located on the basolateral membrane in the intestine (Nemere et al., 1994, 2004).

Pigs fed the 1,500 IU vitamin D/kg diet from cholecalciferol had similar ($P > 0.10$) glucose transport (Fig. 3) compared with pigs fed diets with no supplemental cholecalciferol. We have previously observed an increase in Na^+ -dependent glucose transport in pigs fed a low-P diet; however, pigs in the present study were fed adequate dietary P concentrations (Saddoris et al., 2010). Additionally, chloride secretion (data not shown) was not different between treatment groups ($P < 0.10$), indicating all tissues were still viable at the end of the experimental period in the Ussing chambers.

Gene expression of NaPi-IIb mRNA (Fig. 4) was not different ($P > 0.10$) between pigs fed no supplemental cholecalciferol and pigs fed diets supplemented with 1,500 IU/kg vitamin D from cholecalciferol. These data concur with the P uptake data in which no differences were detected between treatment groups. Xu et al. (2002) reported a 2-fold increase in NaPi-IIb mRNA expression in young, vitamin D adequate rats s.c. injected with $1,25(\text{OH})_2\text{D}_3$. However, this may be due to the method of vitamin D administration, i.m. injection of $1,25(\text{OH})_2\text{D}_3$ compared with dietary cholecalciferol addition, as injecting animals with $1,25(\text{OH})_2\text{D}_3$ would have allowed the hormone access to the basolateral membrane proteins, VDR and MARRS.

Calbindin D(9k) is a calcium-binding protein involved with intestinal Ca absorption, and is transcriptionally regulated by $1,25(\text{OH})_2\text{D}_3$. Krisinger et al. (1991) reported injecting $1,25(\text{OH})_2\text{D}_3$ in vitamin D-deficient rats stimulated calbindin D9k mRNA expression in the intestine. Additionally, dietary vitamin D supplementation to vitamin D-depleted rats also resulted in stimulation calbindin D9k expression compared with negative control rats (Brown et al., 2005). In the current experiment, calbindin D9k expression was numerically increased as the concentration of vitamin D was increased in the diet, but this difference was not significant ($P > 0.10$). However, since we had no vitamin D-deficient control group for comparison, it is impossible to determine if dietary vitamin D supplementation stimulates calbindin D9k mRNA expression in the small intestine of pigs with deficient vitamin D status. However, supplementing dietary vitamin D from cholecalciferol at concentrations exceeding the requirement by 3-fold failed to

Table 3. The effect of dietary vitamin D supplementation of pig performance¹

Vitamin D supplementation, IU/kg diet	0	1,500	P-value
Item			
Initial weight (day 0), kg ²	10.29 $\pm$ 0.26	9.972 $\pm$ 0.37	0.952
Final weight, kg	14.46 $\pm$ 0.29	14.44 $\pm$ 0.40	0.952
ADFI, kg/d	1.213 $\pm$ 0.03	1.230 $\pm$ 0.03	0.836
ADG, kg/d	0.386 $\pm$ 0.007	0.408 $\pm$ 0.01	0.986

¹Means $\pm$ SE, $n = 20$ for 0 IU/kg and $n = 11$ for 1,500 IU/kg.

²Day 0 refers to the day pigs began consuming the various dietary treatments. Data were transformed to achieve normality by y^2 transformation. Statistics were conducted on transformed data but actual means and SE are presented.

stimulated calbindin D9k mRNA expression in the intestine compared with pigs fed at the vitamin D requirement.

Results of the current study show that $1,25(\text{OH})_2\text{D}_3$ stimulates Na^+ -dependent P uptake in a Caco-2 cell model. However, the addition of 1,500 IU/kg vitamin D₃ from cholecalciferol failed to stimulate active P absorption in the small intestine compared with pigs fed diets with no supplemental cholecalciferol. Supplementing diets with a cholecalciferol concentration of 1,500 IU/kg vitamin D provided no benefit in improving the efficiency of P absorption in the small intestine compared with pigs fed diets with no supplemental cholecalciferol. Both the Caco-2 cells and the intestine were bathed in a P-free media prior to evaluation of P transport in the Ussing chambers, so it is unlikely that this would have affected the P status of each model enough to alter how each responded to the addition of 10 nm of phosphate. Since pigs fed either 0 or 1,500 IU/kg vitamin D ended the trial with similar plasma $25(\text{OH})\text{D}_3$ concentrations, circulating $1,25(\text{OH})_2\text{D}_3$ concentrations also may have been equivalent, and hence similar active P transport was detected in the intestine. To directly compare the results of the in vitro and in vivo trial, a difference in circulating $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ concentrations would need to be verified in the in vivo model between cholecalciferol-supplemented and unsupplemented groups. Overall, the Caco-2 cell line may provide a cost-effective model to study intestinal P transport and the mechanisms involved. Additionally, we have successfully developed a low status vitamin D, normocalcemic, pig model by feeding an adequate Ca, vitamin D-deficient milk replacer to early weaned pigs for a 4-wk period. This provides a model for proper investigation of the role of vitamin D metabolites in both Ca and P absorption in the small intestine. However, further research will need to be conducted to investigate if vitamin D₃ supplementation from cholecalciferol is capable of stimulating plasma $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ concentrations in situations where the unsupplemented group does not regain adequate plasma $25(\text{OH})\text{D}_3$ concentrations and to determine if cholecalciferol is capable of stimulating Na^+ -dependent phosphate uptake in pigs fed a low-P diet. Understanding the mechanisms involved in regulating active P absorption may lead to nutritional practices that increase the efficiency of dietary P utilization and reduce P excretion.

Conflict of interest statement. The authors declare no real or perceived conflicts of interest.

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