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Original research article

# Vitamin $D_3$ increased intestinal *Na/Pi-IIb* and *CYP27B1* mRNA level in rats fed low-phosphorus diets

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ABSTRACT

The objective of the study was to determine the role of vitamin $D_3$ ($VD_3$) in regulating adaptation and mechanism of rats to low-phosphorus (P) diets. Rats were assigned to 4 diets containing 0.2%, 0.4%, 0.6%, or 0.8% P consisting of 5 replicate cages with 6 rats per replicate cage and fed for 7 days. Four rats from each replicate cage were treated with ethane-1-hydroxy-1,1-diphosphonicacid, tetrasodium salt (EHDP) and 2 rats remained untreated. Twelve hours prior to preparation on d 7, two of the EHDP-treated rats received an intraperitoneal injection of $VD_3$ [1,25-$(OH)_2D_3$] at 600 ng per kg body weight, while two rats did not receive the injection. Rats that did not receive $VD_3$ injection had decreased ($P < 0.001$) P absorption, but injection of $VD_3$ resulted in increased ($P < 0.001$) absorption. The effect of $VD_3$ injection was greater ($P < 0.001$) for rats fed 0.2% P diet than rats fed 0.8% P diet in ileum. Sodium dependent phosphate cotransporter type IIb (*Na/Pi-II b*) and 25-hydroxyvitamin D 1-α hydroxylase (*CYP27B1*) mRNA level showed the same trend with P absorption. Serum concentration of $VD_3$ and 1α-hydroxylase activity in rats fed 0.2% P diet were lower than those fed 0.8% P diet. The injection of $VD_3$ increased ($P < 0.001$) serum concentration of $VD_3$ and 1α-hydroxylase activity. Thus, $VD_3$ increased *Na/Pi–IIb* and *CYP27B1* mRNA level and improved serum concentration of $VD_3$ and 1α-hydroxylase activity in rats fed low-P diets.



## 1. Introduction

Phosphorus (P) plays an important role in animal metabolism, however, the absorption and regulation of P is not entirely clear, which could affect estimates of P requirement and subsequently P excretion (Fang et al., 2007; Yang et al., 2007; Ruan et al., 2007). Sodium phosphate co-transporter type IIb (Na/Pi-IIb) is the main co-transporter protein involved in P absorption in the small intestine. Dietary P concentration, vitamin $D_3$ ($VD_3$) and hormones are the main factors that co-regulate P absorption. Low-P diets stimulate an increase in *Na/Pi-IIb* mRNA along the entire small intestine and thus dietary P concentration affects P absorption (Wang and Yin, 2009). Vitamin $D_3$ plays a central role in regulating P absorption and homeostasis, 25-$(OH)D_3$ and 1,25-$(OH)_2D_3$ are the two biological activated form of $VD_3$, and 1,25-$(OH)_2D_3$ had the best activity. There are two theories about the mechanism of $VD_3$ regulation of Na/Pi transport: one is the non-gene theory while the other is the gene theory. The non-gene theory proposes that $VD_3$ probably increases the combined sites between sodium and phosphate ions and the vector, or changes Na/Pi-IIb protein expression. Hildmann et al. (1982) demonstrated that $VD_3$ increased the expression of Na/Pi co-transporter proteins in the intestinal apical membrane of VD-deficient rabbits. In contrast, the gene theory proposes that stimulation of intestinal Na/Pi co-transport by a low-P diet or $VD_3$ is mediated via an increase in type IIb transporter expression in Brush-border membrane vesicles (BBMV) (Katai et al., 1999). But the exact mechanisms behind the role of $VD_3$ in adaptation to low phosphate or *Na/Pi-IIb* mRNA level remain obscure. Thus, the goal of the present study was to

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determine the role and mechanism of $VD_3$ regulation P absorption under the low-P diets.

## 2. Materials and methods

### 2.1. Animals and experimental design

A total of 120 male Wister rats, aged 21 d, were assigned to 4 diets consisting of 5 replicate cages with 6 rats per replicate cage. The 4 diets were fed for 7 days and contained 0.2% (very low-P), 0.4% (low-P), 0.6% (normal-P) or 0.8% (high-P) P. Cause of 0.6% P is the normal P requirement for rat, so it was defined as the control group. All rats were fed *ad libitum* and an ambient temperature of 28 ± 1°C and relative humidity of 65%–70% were maintained (Ren et al., 2014). The rat cages were 15 cm (H) × 22 cm (W) × 30 cm (L). Two of the 6 rats in each replicate cage were fed diets with normal $VD_3$ and these were designated as a control group (C group). The remaining 4 rats in each replicate cage, also fed diets with normal $VD_3$, but were injected daily for the first 6 days of the study with ethane-1-hydroxy-1,1-diphosphonicacid, tetrasodium salt (EHDP, purity ≥ 78.5%, Shangdong, China) at 40 mg/(kg·d). The EHDP is known to reduce the circulating levels of $VD_3$, and it was used to hinder $VD_3$ metabolism. Twelve hours (at about the time gastric food would have been completely emptied) prior to preparation on d 7, 2 of the EHDP-treated rats received an intraperitoneal injection of $VD_3$ [1,25-$(OH)_2D_3$, purity ≥ 99.5% , Sigma, USA] at 600 ng per kg body weight and designated injected group (I group). Two remaining EHDP-treated rats were not injected with $VD_3$ but treated with EHDP and designated as the restricted group (R group). On the morning of d 7, all rats were killed by stabbing the eyes to determine calcium (Ca) and P concentrations of the bones and blood, *Na/Pi-IIb*, *CYP27B1* mRNA level and P absorption. The nutritional levels of the diets were based on rat nutrition standards and are showed in Table 1. All protocols used in the study were approved by the Hunan Agriculture University Animal Care and Use Committee.

**Table 1**
Composition and nutrient levels of the basal diets (air-dry basis).

| Item | Treatments | | | |
|---|---|---|---|---|
| | 0.2% P | 0.4% P | 0.6% P | 0.8% P |
| Ingredients, % | | | | |
| Corn starch | 60 | 60 | 60 | 60 |
| Soybean meal | 34 | 34 | 34 | 34 |
| Lysine·HCl | 0.3 | 0.3 | 0.3 | 0.3 |
| Limestone | 0.5 | 0.5 | 0.5 | 0.5 |
| $CaHPO_4·2H_2O$ | 0 | 1.17 | 2.34 | 3.51 |
| Choline | 0.25 | 0.25 | 0.25 | 0.25 |
| NaCl | 0.3 | 0.3 | 0.3 | 0.3 |
| Vitamin premix[1] | 0.1 | 0.1 | 0.1 | 0.1 |
| Mineral premix[2] | 0.1 | 0.1 | 0.1 | 0.1 |
| Zeolite powder | 4.45 | 3.28 | 2.11 | 0.94 |
| Total | 100 | 100 | 100 | 100 |
| Nutrient levels[3], % | | | | |
| DE, MJ/kg | 13.81 | 13.81 | 13.81 | 13.81 |
| CP | 15 | 15 | 15 | 15 |
| Ca | 0.28 | 0.55 | 0.82 | 1.09 |
| P | 0.2 | 0.4 | 0.6 | 0.8 |
| Lysine | 1.2 | 1.2 | 1.2 | 1.2 |
| Ca:P | 1.33:1 | 1.37:1 | 1.36:1 | 1.36:1 |

[1] Provided the following for per kilogram diet: vitamin A, 7,000 IU; vitamin E, 5 mg; vitamin K, 5 mg; vitamin B complex, 110 mg; biotin, 0.2 mg; folic acid, 6 mg.
[2] Provided the following for per kilogram diet: iron, 120 mg; copper, 8 mg; zinc, 30 mg; manganese, 75 mg; selenium, 0.05 mg; iodine, 0.05 mg.
[3] The DE is calculated value, Other indicators are measured value.

### 2.2. Definition of VD-deficient rats

A pre-trial was performed to determine Ca and $VD_3$ concentrations in the blood before the experiment. The mean serum Ca level for all VD-deficient rats used in this study was 52.4 ± 2.9 mg/L, the plasma concentrations of $VD_3$ were below 5 μg/L as suggested by Hildmann et al. (1982).

### 2.3. Sample collection and analyses

All rats were killed and the thigh bone, blood, small intestines, proximal tubule and kidneys were collected. The cartilage was removed from the thigh bone and the Ca and P contents were determined. Blood was centrifuged (TD3, Xiangyi company, China) for 15 min at 3,000 × *g* under the normal temperature to obtain serum for biochemical analysis (Yin et al., 2010). The contents of the small intestine were removed and rinsed in ice-cold 0.9% saline. Small intestine segments containing the ileum, jejunum and duodenum (with every intestinal segment ~ 8 cm) were excised and determined *Na/Pi-IIb* mRNA level and P absorption. Kidneys were collected to determined *CYP27B1* mRNA level and P absorption. The samples were stored in liquid nitrogen for further analysis.

### 2.4. Preparation of BBMV and P transport measurement

Brush border membrane vesicles were prepared from the small intestine and kidneys ($n = 5$) by differential centrifugation (Speed Refrigerated Centrifuge, Hitachi SCR20BC, Japan Tsushima) and magnesium ion ($Mg^{2+}$) precipitation method, as described by Biber et al. (2007). Briefly, the mucosal scrapings were re-suspended at concentrations of 0.7–1.1 g/10 mL in a volume of 40 mL before 1:6 dilutions and homogenization, and $MgC1_2$ was used for precipitation. The first, second and third centrifugation (Hitachi SCR20BC, Shimadzu Corporation, Japan) were carried out at 8,000 × *g* for 15 min, 21,000 × *g* for 30 min and 27,000 × *g* for 40 min under 4°C, respectively. Membranes were re-suspended in 300 mmol/L mannitol, 20 mmol/L HEPES/tris (pH 7.4) with a concentration of 3–8 mg total protein as described by Bradford (1976). Membrane purity was assessed by measuring the activities of alkaline phosphatase (ALP) and $Na^+$, $K^+$ adenosine triphosphataseas described by Mircheff and Wright (1976). Absorption of phosphate was measured using a modified rapid filtration technique (radio-labeled $^{32}P$) as described by Schroder et al. (1998). After 20 μL of BBMV suspension was added to 80 μL of incubation solution (100 mmol/L sodium chloride [NaC1], 100 mmol/L mannitol, 20 mmol/L N-(2-hydroxyethyl) Pperazine-N′-2-ethane sulphonic acid [HEPES]/Tris, and 0.1 mmol/L potassium phosphate [$KH_2PO_4$] pH 7.4) containing 1 μCi$^{32}P$, the mixture was incubated at 25°C for 5 min. Transport was terminated by rapid dilution with 1 mL of an ice-cold solution (100 mmol/L mannitol, 20 mmol/L HEPES/Tris, 0.1 mmol/L $KH_2PO_4$, 20 mmol/L magnesium phosphate [$MgSO_4$], and 100 mmol/L choline chloride [$C_5H_{14}NOC1$], pH 7.4). The solution was centrifuged (Hitachi SCR20BC, Shimadzu Corporation, Japan) at 6,142 × *g* for 5 min, then the supernatant was discarded and 1 mL of formaldehyde solution and 0.36 mL liquid scintillation were added to the precipitation. The reaction mixture was immediately transferred to a β-Radioactive instrument (β-Radioactive Liquid Scintillation Instrument, 1450 Microbeta, Perkin Elmer) to determine the radiation intensity. At the same time, the total radiation intensity of the 80 μL of transfer solution was analyzed. The transport rate of phosphate into BBMV was measured as described previously by Laemmli (1970) at 25°C in the presence of inwardly directed gradients of 100 mmol/L NaCl or 100 mmol/L KCl. The P absorption was determined after 5 min.

### 2.5. Real-time PCR

Total mRNA was extracted from the small intestines and kidneys. Sodium dependent phosphate cotransporter type IIb (Gene ID: AF157026) and β-actin (Gene ID: NM 031144) primers were designed using rat CDS conserved sequences (Liu et al., 2012). Primers were synthesized by Shanghai Biochemical Technology Company. The PCR amplified reaction system for *Na/Pi-IIb* contained 5 μL 1 × qPCR mix, 0.3 μL sense or anti-sense primer (10 μmol/L), 0.5 μL DNA solution, 0.2 μL of 50 × ROX, and distilled water for a final volume of 10 μL. The reaction conditions were as follows: initial denaturation for 60 s at 95°C, denaturation for 15 s at 95°C, extension 15 s at 60°C, and a stop temperature of 60°C, for 40 recycles.

The PCR reaction condition for *Na/Pi-IIa* (Gene ID:733703) was as follows: 5 μL 1 × qPCR mix, 0.5 μL sense or anti-sense primer (10 μm), 0.5 μL DNA solution, 0.2 μL of 50 × ROX, and distilled water were added to a final volume of 10 μL. The Real Time PCR system with thermocycline setting of 50°C for 2 min, 95°C for 10 min, and 40°C reps of 95°C for 15 s followed by 60°C for 1 min (Yao et al., 2012). The PCR reaction condition for *CYP27B1* (Gene ID: 22588163) was as follows: 5 μL 2 × qPCR mix, 0.3 μL sense or anti-sense primer (10 μm), 0.5 μL DNA solution, 0.3 μL of 50 × ROX, and distilled water volume of 10 μL. The reaction conditions were as follows : 94°C-5 min → (94°C-30 s → 59°C-30 s → 72°C-30 s) × 35 → 72°C-10 min. The details of the reaction are showed in Table 2.

### 2.6. Statistical analysis

The mRNA level was calculated with $2^{-\Delta\Delta Ct}$ by relative ratio. All data were analyzed using the General Linear Model procedure of SAS9.1. Cage served as the experimental unit for all analysis. The model for this analysis included 4 dietary P levels (very low [0.2% P], low [0.4% P], normal [0.6% P] and high [0.8% P]), 3 $VD_3$ status (normal $VD_3$ [C group], normal $VD_3$ + EHDP injection [R group], normal $VD_3$ + EHDP injection + $VD_3$ injection prior slaughter [I group]), and the interaction between dietary P levels and $VD_3$ status (P levels × $VD_3$ treatments) in a split-plot with P levels as the whole plot and $VD_3$ status as subplot. Possible difference test was used to separate means. A probability level of 0.05 was considered statistically significant.

## 3. Results

### 3.1. Effects of $VD_3$ treatment and dietary P levels on Ca and P deposition and biochemical indices

The effects of $VD_3$ and dietary P levels on Ca and P deposition and biochemical indices are presented in Table 3. Vitamin $D_3$ affected ($P < 0.001$) bone Ca, P and serum Ca levels. Bone Ca and P of rats fed diet with 0.2% and 0.4% P levels showed lower ($P < 0.05$) values than those of 0.6% and 0.8% P groups. Rats that were not injected with $VD_3$ (R group) showed the lowest ($P < 0.05$) concentration of bone Ca, especially for the rats fed low-P diets. Injection of $VD_3$ did not restore bone Ca. Varying dietary P levels also showed no effect on serum P concentration.

Dietary P levels and various $VD_3$ treatments imposed in this study significantly influenced the serum $VD_3$ concentration. The serum $VD_3$ concentration in rats injected $VD_3$ after EHDP treatment was 3 times more than that in the rats injected only with EHDP. There was no effect of dietary P on 1α-hydroxylase activity in the R group. Injection of $VD_3$ increased ($P < 0.001$) proximal tubule 1α-hydroxylase activity, especially for rats fed diets containing 0.2% and 0.4% P. The fibroblast growth factor 23 (FGF23) content of rats fed 0.2% P diet with $VD_3$ (I group) was significantly increased.

### 3.2. Na/Pi-IIb and CYP27B1 mRNA level

Dietary P content and $VD_3$ influenced ($P < 0.001$) the *Na/Pi-IIb* mRNA level as shown in Fig. 1. The *Na/Pi-IIb* mRNA level of VD-restricted rats were the least, while injection of $VD_3$ increased ($P < 0.01$) *Na/Pi-IIb* mRNA level, especially for rats in 0.2% P treatment in the ileum (Fig. 1A), jejunum (Fig. 1B) and duodenum (Fig. 1C). However, the effects of dietary P and $VD_3$ on *Na/Pi-IIa* mRNA level showed an opposite difference in the kidneys (Fig. 1D). The *Na/Pi-IIa* mRNA level of rats fed the 0.2% P diet was the lowest ($P < 0.01$) in the kidneys, while that of the 0.6% or 0.8% P groups was 30.82% and 28.92% greater ($P < 0.01$) than that of rats fed 0.2% P diet and injection with $VD_3$ prior slaughter (Fig. 1D). These results suggested that the reaction of *Na/Pi-IIb* mRNA level was more sensitive to low-P diets when $VD_3$ is restricted, and injection of $VD_3$ increased the most expression to the 0.2% level diets; but the effects were the opposite in the kidneys, with normal or high P diets giving more mRNA level.

Fig. 2 shows that dietary P level did not have effects on expression of *CYP27B1* mRNA when dietary $VD_3$ was normal, with $VD_3$ restriction giving more on lower P diets when compared with normal or high P diets. When $VD_3$ was injected, the expression of *CYP27B1* mRNA was increased ($P < 0.01$), more was for the 0.2% treatment.

### 3.3. Effects of $VD_3$ and low-P diets on P absorption

Data on the effects of $VD_3$ and low-P diets on P absorption are presented in Fig. 3. Dietary P level and $VD_3$ affected ($P < 0.001$) P absorption. The effects of $VD_3$ on P absorption of any dietary P level were consistent with *Na/Pi-IIb* mRNA. Rats fed normal $VD_3$ or

**Table 2**
The sequences, amplified regions and fragment length of *Na/Pi-IIb* and *CYP27B1* primers.

| Primer name | Sequences of the primer pair[1] | Fragment length, bp |
|---|---|---|
| *Na/Pi-IIb* sense | 5′-CGTCCTCGTCAATCATCGTCAG-3′ | 123 |
| *Na/Pi-IIb* antisense | 5′-GCATAAGTGCCACAATCGTGTT-3′ | |
| *Na/Pi-IIa* sense | 5′-TCTGAGAGTGCTGATGTACCTAAGT-3′ | 120 |
| *Na/Pi-IIa* antisense | 5′-AGGTACTCATCCAACACCAGGTAT-3′ | |
| *CYP27B1* sense | 5′-TGCCTTAAGAGTGGAGCCATATGT-3′ | 118 |
| *CYP27B1* antisense | 5′-TTGGTTTACATGTCGCACGTCTC-3′ | |
| β-actin sense | 5′-CCGTAAAGACCTCTATGCCAACA-3′ | 113 |
| β-actin antisense | 5′-GCTAGGAGCCAGGGCAGTAATCT-3′ | |

*Na/Pi-IIb* = sodium phosphate co-transporter type IIb; *CYP27B1* = 25-hydroxyvitamin D 1-α hydroxylase.
[1] The primers were designed using Primer Expression software Primer Premier 5.

**Table 3**
Dietary P levels and vitamin $D_3$ ($VD_3$) affected Ca and P concentrations of bone and serum.[1]

| Item | 0.2% P | | | 0.4% P | | | 0.6% P | | | 0.8% P | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | R | I | C | R | I | C | R | I | C | R | I | SEM | Dietary P | $VD_3$ | Dietary P × $VD_3$ |
| Bone | | | | | | | | | | | | | | | | |
| Ca, mg/100 g | 76.6 | 40.1* | 33.3* | 79.7 | 32.0* | 34.0* | 105.6 | 115.8 | 64.4* | 116.1 | 119.4 | 84.7* | 3.27 | <0.001 | <0.001 | <0.001 |
| P, mg/100 g | 48.7 | 34.7* | 34.8* | 52.9 | 32.3* | 39.2* | 72.7 | 254.5* | 245.5* | 76.5 | 247.6* | 246.0* | 2.43 | <0.001 | <0.001 | <0.001 |
| Serum | | | | | | | | | | | | | | | | |
| Ca, mg/L | 86.6 | 42.4* | 42.2* | 86.6 | 51.5* | 52.9* | 92.1 | 44.4* | 51.4* | 99.4 | 41.7* | 49.3* | 2.98 | 0.038 | <0.001 | 0.062 |
| P, mg/L | 81.4 | 77.7 | 92.4 | 79.7 | 114.4※ | 91.0 | 96.1 | 92.4 | 96.4 | 99.5 | 90.1 | 99.9 | 5.90 | 0.045 | 0.36 | 0.008 |
| $VD_3$, μg/L | 9.3 | 3.9* | 11.9* | 8.6 | 2.9* | 10.9* | 7.1 | 2.7* | 9.1* | 8.1 | 2.7* | 9.9* | 0.11 | <0.001 | <0.001 | <0.001 |
| 1α-hydroxylase, mU/mg prot | 108.4 | 84.7* | 135.4* | 112.5 | 91.2* | 122.6* | 94.8 | 95.3 | 102.6* | 105.8 | 102.4 | 111.5* | 0.63 | <0.001 | <0.001 | <0.001 |
| FGF23, μg/L | 20.1 | 20.4 | 24.9※ | 19.5 | 21.2 | 25.9※ | 23.6 | 23.9 | 25.4 | 24.8 | 22.4 | 24.3 | 3.02 | 0.035 | 0.016 | <0.001 |

FGF23 = fibroblast growth factor 23.
[1] Data are means of 6 replicates per treatment. C represents control group; R represents restricted $VD_3$ group; I represents group injected with $VD_3$. "※" or "*" means are different from the C group at $^{※}P < 0.01$ and $^{*}P < 0.01$.

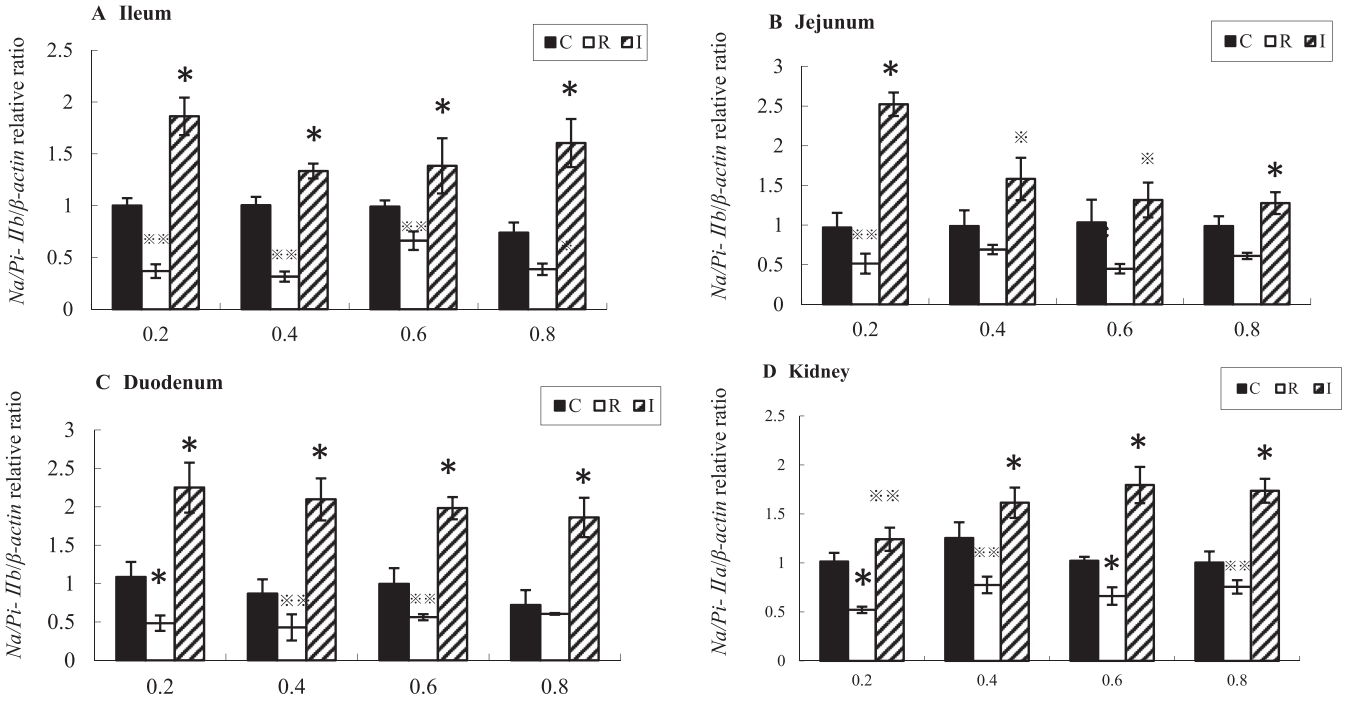


**Fig. 1.** Effects of dietary P (0.2%, 0.4%, 0.6% or 0.8%) and Vitamin $D_3$ ( $VD_3$) on *Na/Pi-IIb* mRNA level in ileum (A), jejunum (B), duodenum (C) and kidneys (D) of rats. Data are means of 6 replicates per treatment. C represents control group; R represents restricted $VD_3$ group; I represents group injected with $VD_3$. "※" and "*" indicate that means are different from that of the C group at $^{※}P < 0.05$, $^{※※}P < 0.01$ and $^{*}P < 0.001$.

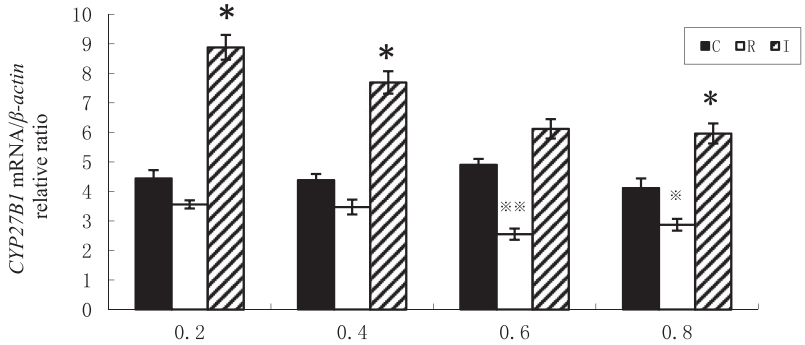


**Fig. 2.** Effect of dietary P (0.2%, 0.4%, 0.6% or 0.8%) and Vitamin $D_3$ ($VD_3$) on *CYP27B1* mRNA level in kidneys. Data are means of 6 replicates per treatment. C represents control group; R represents restricted vitamin D group; I represents group injected with $VD_3$. "※" and "*" indicate that means are different from that of the C group at $^{※}P < 0.05$, $^{※※}P < 0.01$ and $^{*}P < 0.001$.

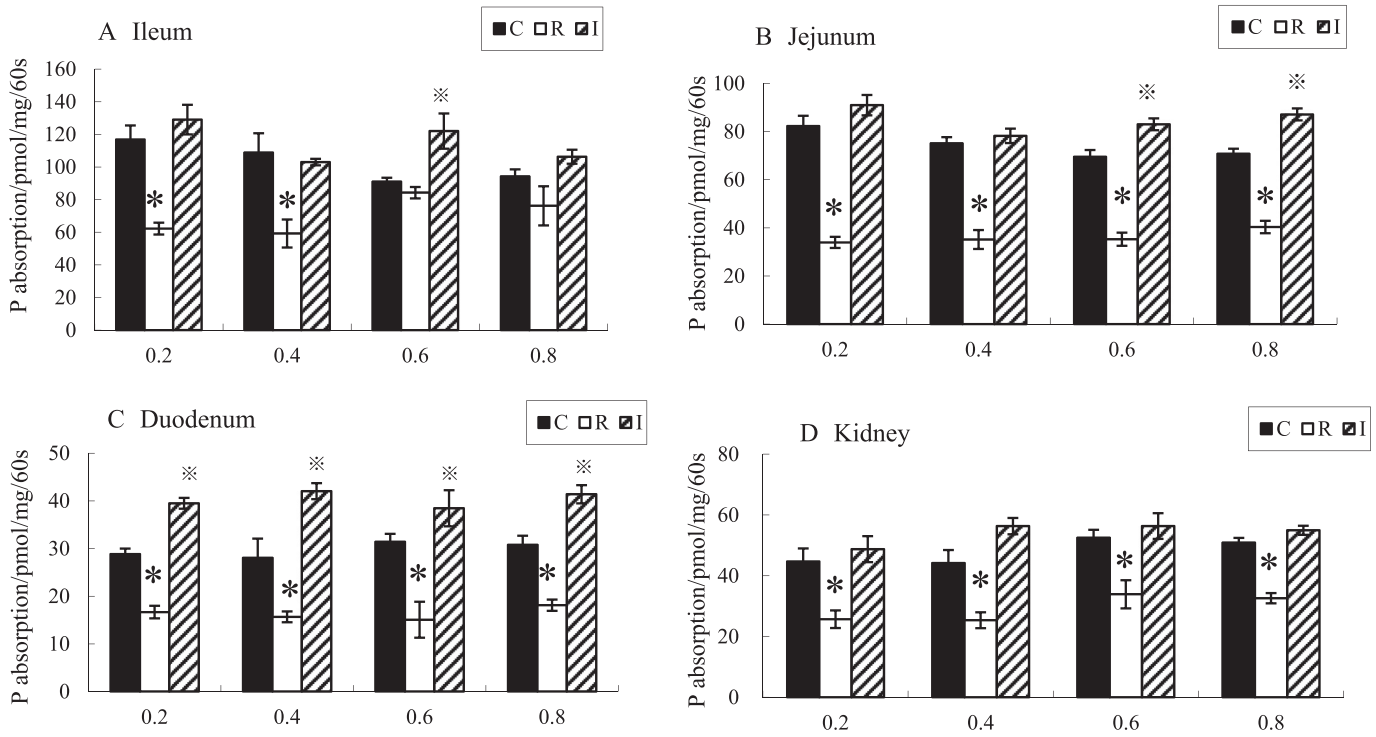


**Fig. 3.** Effects of dietary P (0.2%, 0.4%, 0.6% or 0.8%) and Vitamin $D_3$ ($VD_3$) on P absorption in ileum (A), jejunum (B), duodenum (C) and kidneys (D) of rats. Data are means of 6 replicates per treatment. C represents control group; R represents restricted vitamin D group; I represents group injected with $VD_3$. "※" and "*" indicate that means are different from that of the C group at $^{※}P < 0.05$, $^{※※}P < 0.01$ and $^{*}P < 0.001$.

injected with $VD_3$ had higher ($P < 0.01$) P absorption than VD-restricted rats. More P absorption was observed in the I group, and the greatest for rats fed 0.2% P diet in the ileum. Rats fed a normal P diet had greater ($P < 0.01$) P absorption in small intestine than those fed a low-P diet when $VD_3$ was deficient, but P absorption was greater ($P < 0.01$) in the low P diet treatments when $VD_3$ was injected.

## 4. Discussion and conclusion

### 4.1. Effects of dietary P and $VD_3$ on Ca and P metabolism

Our study showed that regardless of $VD_3$ status, Ca and P of bone increased with dietary P, which is consistent with metabolic drive for mineral homeostasis. Vitamin $D_3$ is an important factor that regulates Ca and P metabolism in small intestines, bones, or kidneys. The $1,25(OH)_2D_3$ is the predominant activated form of $VD_3$ which is converted by $25(OH)_2D_3$ in kidneys. It significantly impacts the metabolism and absorption of Ca and P. Our study showed that low-P diets decreased bone Ca and P concentrations and the effects of $VD_3$ on bone Ca and P occurs in a short time. Vitamin $D_3$ can elevate bone P concentration, and low-P diets may lead to chronic adaptation of P-deprived animals and stimulates $25(OH)_2D_3$ in kidneys converted to $1,25(OH)_2D_3$ (Knowlton et al., 2004; Huber et al., 2006), which is also the reason of an increase of serum $VD_3$. Our study also suggested that $VD_3$ concentration of serum showed no difference between 4 P levels when $VD_3$ was restricted; however, it elevated after $VD_3$ injection, and the effects were seen most clearly under low P compared with normal dietary P.

### 4.2. Dietary P effects on Na/Pi-IIb expression and P absorption

Dietary P regulates P absorption and low P stimulates P absorption in small intestine (Muscher et al., 2007; Saddoris et al., 2010). In our study, *Na/Pi-IIb* mRNA level of the 0.2% P group was greater than those of the 0.6% or 0.8% P groups when $VD_3$ was administered to $VD_3$ restricted groups. This partly agreed with the mechanism of P absorption under low-P conditions (Virkki et al., 2007; Giral et al., 2009). Passive diffusion is a major means to P absorption when inorganic P concentration is high. When inorganic P concentration decreased, *Na/Pi-II* co-transporters play an important role for P absorption in small intestine or kidneys. These co-transporters dominate 75%–90% of total inorganic P transport under low-P conditions (Segawa et al., 2002, 2004, 2011; Villa-Bellosta et al., 2009).

### 4.3. Regulation of phosphate absorption under low-P feeding

This current study suggested that VD-restricted rats absorbed the lowest P in small intestine. Injection of $VD_3$ significantly increased P absorption, especially for 0.2% treatment. However, the interaction effects of diet P and $VD_3$ on P absorption in kidneys was different from that in small intestine. When rats fed normal or high-P diet, P absorption was greater than the rats fed low-P diets under normal $VD_3$ situation. Consistent with that, $VD_3$ injection into rat fed normal or high-P diets increased P absorption.

In this study, low-P diets stimulated serum $VD_3$ concentration when $VD_3$ was at physiological levels. However, serum $VD_3$ concentration significantly increased with the administration of $VD_3$ to restricted rats. Moreover, low-P treatment increased serum $VD_3$ concentration most. It is possible that dietary P restriction

provoked the synthesis of $VD_3$, which is consistent with low-P stimulation of $VD_3$ synthesis (Murer et al., 2004).

The non-gene theory of the $VD_3$ regulation of the *Na/Pi* proposes that $VD_3$ probably increases the combined sites between the sodium P ions to the vector, or changes *Na/Pi-IIb* protein and mRNA level (Capuano et al., 2005). The gene theory holds that $VD_3$ participates in regulating low-P adaptation of the small intestines, it can alter small intestine membrane composition and improve membrane mobility (Marks et al., 2006). In this study, expression of *Na/Pi-IIb* mRNA was not affected by P treatments when dietary $VD_3$ was restricted; however, it significantly increased after $VD_3$ was injected. This is consistent with mechanisms of P absorption that rats fed low-P diets had the greatest expression of *Na/Pi-IIb* mRNA in small intestine (Saddoris et al., 2010). These results suggested that $VD_3$ probably increased the P absorption rate by increasing the expression of *Na/Pi-IIb* mRNA when body P status was low. Our results are also supported by Xu et al. (2003), whose study showed that *Na/Pi-IIb* mRNA level was 2.5-fold after injection of $VD_3$ to VD-restricted rats. However, the results differ with Hattenhauer et al. (1999) who observed that *Na/Pi-IIb* mRNA showed no changes, but that the co-transporter protein expression increased after 12 h injection of $VD_3$ to VD-restricted rats. The differences are likely related to differences in the age of experimental animals. The mechanism of $VD_3$ increase of P absorption is in agreement with the gene theory. In our study, $VD_3$ promoted P absorption by increasing *Na/Pi-IIb* mRNA level. The trend obtained for *CYP27B1* mRNA level, 1α-hydroxylase activity and FGF23 in this study supported the above conclusions. 25-hydroxyvitamin D 1-α hydroxylase is a gene encoded 25-hydroxyvitamin D 1a-hydroxylase, which converts 25-$(OH)D_3$ to the biological active form of $1,25(OH)_2D_3$. The current study showed low-P diets under restricted VD decreased phosphorus absorption, which was also supported by the result of *CYP27B1* mRNA level and 1a-hydroxylase activity. Fibroblast growth factor 23 decreases serum $VD_3$ concentrations by suppressing *CYP27B1* mRNA (Alon, 2011; Bacchetta et al., 2011; Chanakul et al., 2013). Our study showed that FGF23 was significantly reduced under low dietary P.

Results of the present study are consistent with the gene theory and showed that when $VD_3$ is low or normal, reduced dietary P levels do not stimulate P absorption and mRNA level of *Na/Pi-IIb*. However, $VD_3$ injection of rats fed low-P diets results in increased P absorption to levels higher than in rats on high-P diet. Regulation of P absorption by $VD_3$ in rats fed low-P diets is related to the observation that $VD_3$ increased *Na/P—IIb*, *CYP27B1* mRNA levels, serum $VD_3$ concentration and 1α-hydroxylase activity.

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