Original research article
Vitamin D3 increased intestinal Na/Pi-IIb and CYP27B1 mRNA level in rats fed low-phosphorus diets
Manhu Cao, Rejun Fang, Juan Chen, Jianhua He*
College of Animal Science and Technology, Hunan Agriculture University, Changsha 410128, China

ABSTRACT
The objective of the study was to determine the role of vitamin D3 (VD3) 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-diphosphonic acid, 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 VD3 [1,25-(OH)2D3] at 600 ng per kg body weight, while two rats did not receive the injection. Rats that did not receive VD3 injection had decreased (P < 0.001) P absorption, but injection of VD3 resulted in increased (P < 0.001) absorption. The effect of VD3 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 co-transporter type IIb (Na/Pi-IIb) and 25-hydroxyvitamin D 1-α-hydroxylase (CYP27B1) mRNA level showed the same trend with P absorption. Serum concentration of VD3 and 1α-hydroxylase activity in rats fed 0.2% P diet were lower than those fed 0.8% P diet. The injection of VD3 increased (P < 0.001) serum concentration of VD3 and 1α-hydroxylase activity. Thus, VD3 increased Na/Pi-IIb and CYP27B1 mRNA level and improved serum concentration of VD3 and 1α-hydroxylase activity in rats fed low-P diets.

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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 D3 (VD3) 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 D3 plays a central role in regulating P absorption and homeostasis, 25-(OH)D3 and 1,25-(OH)2D3 are the two biological activated form of VD3, and 1,25-(OH)2D3 had the best activity. There are two theories about the mechanism of VD3 regulation of Na/Pi transport: one is the non-gene theory while the other is the gene theory. The non-gene theory proposes that VD3 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 VD3 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 VD3 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 VD3 in adaptation to low phosphate or Na/Pi-IIb mRNA level remain obscure. Thus, the goal of the present study was to...
determine the role and mechanism of VD₃ regulation P absorption under the low-P diets.

2. Materials and methods

2.1. Animals and experimental design

A total of 120 male Wistar 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 designated as a 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 VD₃ and these were designated as a control group (C group). The remaining 4 rats in each replicate cage, also fed diets with normal VD₃, but were injected daily for the first 6 days of the study with ethane-1-hydroxy-1,1-diphosphonic acid, tetrasodium salt (EHDP, purity > 78.5%, Shandong, China) at 40 mg/(kg d). The EHDP is known to reduce the circulating levels of VD₃, and it was used to hinder VD₃ metabolism. Twelve hours (at 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 VD₃[1,25-(OH)₂D₃, 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 VD₃ 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-Ⅱ, CYP27B1 mRNA level and P absorption. The nutritional levels of the diets were based on rat nutrition standards and are shown in Table 1. All protocols used in the study were approved by the Hunan Agriculture University Animal Care and Use Committee.

2.2. Definition of VD-deficient rats

A pre-trial was performed to determine Ca and VD₃ 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 VD₃ were below 5 μg/L, as suggested by Hildemann 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 weighed. The proximal tubule and kidneys were collected. The cartilage was frozen at −80°C and stored 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. 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 weighed. The proximal tubule and kidneys were collected. The cartilage was frozen at −80°C and stored.

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 PCR20BC, Japan Tsubosaka) and magnesium ion (Mg²⁺) precipitation method, as described by Biber et al. (2007). Briefly, the mucosal scrapings were resuspended at concentrations of 0.7–1.1 g/10 mL in a volume of 40 mL before 1:6 dilutions and homogenization, and MgCl₂ was used for precipitation. The first, second and third centrifugation (Hitachi PCR20BC, 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 triphosphatase described by Mircheff and Wright (1976). Absorption of phosphate was measured using a modified rapid filtration technique (radio-labeled ³²P) 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 [NaCl], 100 mmol/L mannitol, 20 mmol/L N-[(-hydroxyethyl) P-perazine-N''-ethyl sulphonic acid (HEPES)/Tris, and 0.1 mmol/L potassium phosphate [KH₂PO₄] pH 7.4) containing 1 μCi³²P, 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/Lmannitol, 20 mmol/L HEPES/Tris, 0.1 mmol/LKH₂PO₄, 20 mmol/L magnesium phosphate [MgSO₄], and 100 mmol/L choline chloride [C₃H₇NOCl], pH 7.4). The solution was centrifuged (Hitachi PCR20BC, Shimadzu Corporation, Japan) at 6,142 × g for 5 min, then the supernatant was discarded and 1 mL of formamide 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 radioactivity. At the same time, the total radioactivity 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.

### Table 1

| 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    |
| CaHPO₄·2H₂O      |            | 0.1    | 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 premix¹  |            | 0.1    | 0.1    | 0.1    | 0.1    |
| Mineral premix²  |            | 0.1    | 0.1    | 0.1    | 0.1    |
| Zeolite powder   |            | 4.45   | 3.28   | 2.11   | 0.94   |
| Total            |            | 100    | 100    | 100    | 100    |
| Nutrient levels¹, % |        |        |        |        |        |
| 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.
2.5. 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 amplification 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 thermostyrene 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 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 shown in Table 2.

2.6. Statistical analysis

The mRNA level was calculated with 2−ΔΔCt 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 VD3 status (normal VD3 [C group], normal VD3 + EHDP injection [R group], normal VD3 + EHDP injection + VD3 injection prior slaughter [I group]), and the interaction between dietary P levels and VD3 status (P levels × VD3 treatments) in a split-plot with P levels as the whole plot and VD3 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 VD3 treatment and dietary P levels on Ca and P deposition and biochemical indices

The effects of VD3 and dietary P levels on Ca and P deposition and biochemical indices are presented in Table 3. Vitamin D3 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 VD3 (R group) showed the lowest (P < 0.05) concentration of bone Ca, especially for the rats fed low-P diets. Injection of VD3 did not restore bone Ca. Varying dietary P levels also showed no effect on serum P concentration.

Dietary P levels and various VD3 treatments imposed in this study significantly influenced the serum VD3 concentration. The serum VD3 concentration in rats injected VD3 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 VD3 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 VD3 (I group) was significantly increased.

3.2. Na/Pi-IIa and CYP27B1 mRNA level

Dietary P content and VD3 influenced (P < 0.001) the Na/Pi-IIa mRNA level as shown in Fig. 1. The Na/Pi-IIa mRNA level of VD3-restricted rats were the least, while injection of VD3 increased (P < 0.01) Na/Pi-IIa 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 VD3 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 VD3 prior slaughter (Fig. 1D). These results suggested that the reaction of Na/Pi-IIa mRNA level was more sensitive to low-P diets when VD3 is restricted, and injection of VD3 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 VD3 was normal, with VD3 restriction giving more on lower P diets when compared with normal or high P diets. When VD3 was injected, the expression of CYP27B1 mRNA was increased (P < 0.01), more was for the 0.2% treatment.

3.3. Effects of VD3 and low-P diets on P absorption

Data on the effects of VD3 and low-P diets on P absorption are presented in Fig. 3. Dietary P level and VD3 affected (P < 0.001) P absorption. The effects of VD3 on P absorption of any dietary P level were consistent with Na/Pi-IIa mRNA. Rats fed normal VD3 or

Table 2

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

| Primer name          | Sequences of the primer pair | Fragment length, bp |
|----------------------|------------------------------|---------------------|
| Na/Pi-IIb sense      | 5′-CGCTCTGTCGTAATTACATGGTCA-3′ | 123                 |
| Na/Pi-IIb antisense  | 5′-GCATAAGTGCCAAAATCCTGTT-3′ | 120                 |
| Na/Pi-IIb sense      | 5′-TTCGAGATGCTCGATTGCTCAGT-3′ | 118                 |
| Na/Pi-IIb antisense  | 5′-AGGTAATCATTCCAAACCGAGTAT-3′ | 113                 |
| CYP27B1 sense        | 5′-TGCTTAAAGACTGGACGCATATTT-3′ | 112                 |
| CYP27B1 antisense    | 5′-TCTGTTTAAGTCTGCAGCTTCTC-3′ | 110                 |
| β-actin sense        | 5′-CCGTTAAGACCTCTTATGCCAAA-3′ | 108                 |
| β-actin antisense    | 5′-CTTGGAGGGCCAGCGGAGTATT-3′ | 105                 |

Na/Pi-IIb = sodium phosphate co-transporter type IIb; CYP27B1 = 25-hydroxvitamin D 1α-hydroxylase.

The primers were designed using Primer Expression software Primer Premier 5.
Table 3
Dietary P levels and vitamin D₃ (VD₃) 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 |
|-----------------------------|--------|--------|--------|--------|---------|
| Bone                        |        |        |        |        |         |
| Ca, mg/100 g                |        |        |        |        |         |
| C                           | 76.6   | 40.1*  | 33.3*  |        |         |
| CR                          | 79.7   | 32.0   |        |        |         |
| C I                         | 105.6  | 115.8  | 64.4*  |        |         |
| SEM                         |        |        |        |        |         |
| Bone                        |        |        |        |        |         |
| Ca, mg/L                    |        |        |        |        |         |
| C                           | 86.6   | 12.4   | 42.2   |        |         |
| R                           | 86.6   | 51.5*  | 91.9   |        |         |
| I                           | 96.1   | 91.0   |        |        |         |
| SEM                         |        |        |        |        |         |
| Bone                        |        |        |        |        |         |
| P, mg/100 g                 |        |        |        |        |         |
| C                           | 81.4   | 77.7   | 92.4   |        |         |
| R                           | 89.7   | 92.7   |        |        |         |
| I                           | 99.1   | 91.1   |        |        |         |
| SEM                         |        |        |        |        |         |
| Bone                        |        |        |        |        |         |
| P, mg/L                     |        |        |        |        |         |
| C                           | 9.3    | 3.9*   | 11.9   |        |         |
| R                           | 8.6    | 2.9    | 10.9*  |        |         |
| I                           | 7.1    | 2.7*   | 9.1*   |        |         |
| SEM                         |        |        |        |        |         |
| Bone                        |        |        |        |        |         |
| VD₃ mg/L                    | 108.4  | 84.7*  | 112.5  |        |         |
| C                           | 94.8   | 95.3   | 102.6  |        |         |
| R                           | 105.8  | 102.4  | 111.5* |        |         |
| I                           | 114.4  | 76.5   | 247.6  |        |         |
| SEM                         |        |        |        |        |         |
| Bone                        |        |        |        |        |         |
| FGF23, μg/L                 |        |        |        |        |         |
| C                           | 20.1   | 20.4   | 19.5   |        |         |
| R                           | 24.9*  | 21.2   | 25.9*  |        |         |
| I                           | 23.6   | 23.9   | 25.4   |        |         |
| SEM                         |        |        |        |        |         |
| Bone                        |        |        |        |        |         |
| FGF23 mRNA                  |        |        |        |        |         |
| C                           |        |        |        |        |         |
| R                           |        |        |        |        |         |
| I                           |        |        |        |        |         |
| SEM                         |        |        |        |        |         |

¹ Data are means of 6 replicates per treatment. C represents control group; R represents restricted VD₃ group; I represents group injected with VD₃. “※” and “*” 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 D₃ (VD₃) 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 VD₃ group; I represents group injected with VD₃. “※” 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 D₃ (VD₃) 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 VD₃. “※” 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 VD3 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 VD3 was deficient, but P absorption was greater ($P < 0.01$) in the low P diet treatments when VD3 was injected.

4. Discussion and conclusion

4.1. Effects of dietary P and VD3 on Ca and P metabolism

Our study showed that regardless of VD3 status, Ca and P of bone increased with dietary P, which is consistent with metabolic drive for mineral homeostasis. Vitamin D3 is an important factor that regulates Ca and P metabolism in small intestines, bones, or kidneys. The 1,25(OH)2D3 is the predominant activated form of VD3 which is converted by 25(OH)2D3 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 VD3 on bone Ca and P occurs in a short time. Vitamin D3 can elevate bone P concentration, and low-P diets may lead to chronic adaptation of P-deprived animals and stimulates 25(OH)2D3 in kidneys converted to 1,25(OH)2D3 (Knowlton et al., 2004; Huber et al., 2006), which is also the reason of an increase of serum VD3. Our study also suggested that VD3 concentration of serum showed no difference between 4 P levels when VD3 was restricted; however, it elevated after VD3 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 VD3 was administered to VD3 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 VD3 significantly increased P absorption, especially for 0.2% treatment. However, the interaction effects of diet P and VD3 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 VD3 situation. Consistent with that, VD3 injection into rat fed normal or high-P diets increased P absorption.

In this study, low-P diets stimulated serum VD3 concentration when VD3 was at physiological levels. However, serum VD3 concentration significantly increased with the administration of VD3 to restricted rats. Moreover, low-P treatment increased serum VD3 concentration most. It is possible that dietary P restriction

Fig. 3. Effects of dietary P (0.2%, 0.4%, 0.6% or 0.8%) and Vitamin D3 (VD3) 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 VD3. “※” and “★★” indicate that means are different from that of the C group at “※” $P < 0.05$, “★★” $P < 0.01$ and “★” $P < 0.001$. 
provoked the synthesis of VD3, which is consistent with low-P stimulation of VD3 synthesis (Murer et al., 2004).

The non-gene theory of the VD3 regulation of the Na/Pi proposes that VD3 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 VD3 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 VD3 was restricted; however, it significantly increased after VD3 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 VD3 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 VD3 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 VD3 to VD-restricted rats. These differences are likely related to differences in the age of experimental animals. The mechanism of VD3 increase of P absorption is in agreement with the gene theory. In our study, VD3 promoted P absorption by increasing Na/Pi-Ⅱb mRNA level. The trend obtained for CYP27B1 mRNA level, 1a-hydroxylase activity and FGF23 in this study supported the above conclusions. 25-hydroxyvitamin D 1a-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)2D3. 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 VD3 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 VD3 is low or normal, reduced dietary P levels do not stimulate P absorption and mRNA level of Na/Pi-Ⅱb. However, VD3 injection of rats fed low-P diets resulted in increased P absorption to levels higher than in rats on high-P diet. Regulation of P absorption by VD3 in rats fed low-P diets is related to the observation that VD3 increased Na/P-Ⅱb, CYP27B1 mRNA levels, serum VD3 concentration and 1α-hydroxylase activity.

Acknowledgments

The study was supported by grants from the Nature Science Foundation (31201810, to M H Cao; 31572419, to R J Fang), Innovation Team Funds Of Hunan University (to J H He), Academic advice and English revision by Professor Olayiwola Adeola of Purdue University are gratefully appreciated.

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