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Age-related alteration of vitamin D metabolism in response to low-phosphate diet in rats

Tsui-Shan Chau[1]†, Wan-Ping Lai[1]†, Pik-Yuen Cheung[1], Murray J. Favus[2] and Man-Sau Wong[1]*

[1]*Central Laboratory of the Institute of Molecular Technology for Drug Discovery and Synthesis, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, PRC*
[2]*Section of Endocrinology, Department of Medicine, The University of Chicago, Chicago, IL 60637, USA*

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The responses of renal vitamin D metabolism to its major stimuli alter with age. Previous studies showed that the increase in circulating 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) as well as renal 25-hydroxyvitamin D_3 1-α hydroxylase (1-OHase) activity in response to dietary Ca or P restriction reduced with age in rats. We hypothesized that the mechanism involved in increasing circulating $1,25(OH)_2D_3$ in response to mineral deficiency alters with age. In the present study, we tested the hypothesis by studying the expression of genes involved in renal vitamin D metabolism (renal 1-OHase, 25-hydroxyvitamin D 24-hydroxylase (24-OHase) and vitamin D receptor (VDR)) in young (1-month-old) and adult (6-month-old) rats in response to low-phosphate diet (LPD). As expected, serum $1,25(OH)_2D_3$ increased in both young and adult rats upon LPD treatment and the increase was much higher in younger rats. In young rats, LPD treatment decreased renal 24-OHase (days 1–7, $P<0·01$) and increased renal 1-OHase mRNA expression (days 1–5, $P<0·01$). LPD treatment failed to increase renal 1-OHase but did suppress 24-OHase mRNA expression ($P<0·01$) within 7 d of LPD treatment in adult rats. Renal expression of VDR mRNA decreased with age ($P<0·001$) and was suppressed by LPD treatment in both age groups ($P<0·05$). Feeding of adult rats with 10 d of LPD increased 1-OHase ($P<0·05$) and suppressed 24-OHase ($P<0·001$) as well as VDR ($P<0·05$) mRNA expression. These results indicate that the increase in serum $1,25(OH)_2D_3$ level in adult rats during short-term LPD treatment is likely to be mediated by a decrease in metabolic clearance via the down-regulation of both renal 24-OHase and VDR expression. The induction of renal 1-OHase mRNA expression in adult rats requires longer duration of LPD treatment than in younger rats.

25-Hydroxyvitamin D_3 1-α hydroxylase: 25-Hydroxyvitamin D_3 24 hydroxylase: Vitamin D receptor: Low-phosphate diet: Adult rats

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D_3, is the major regulator of intestinal Ca and P absorption. Serum $1,25(OH)_2D_3$ level is under stringent control to meet the skeletal requirement of Ca and P, such as during growth, pregnancy, lactation or when the dietary intake of Ca and P is low (Wilz *et al.* 1979; Favus & Tembe, 1992). The major regulators of $1,25(OH)_2D_3$ levels are parathyroid hormone (PTH; Garabedian *et al.* 1972), serum Ca and P (Hughes *et al.* 1975), growth hormone (Spanos *et al.* 1978), insulin-like growth factor I (Gray & Garthwaite, 1985; Nesbitt & Drezner, 1993; Condamine *et al.* 1994), oestrogen (Pike *et al.* 1978) and $1,25(OH)_2D_3$ itself (Dick *et al.* 1990; Jones *et al.* 1998). Serum $1,25(OH)_2D_3$ levels decline with age (Armbrecht *et al.* 1982; Clemens *et al.* 1986). The decline in serum $1,25(OH)_2D_3$ levels with ageing is thought to be one of the contributing factors in the pathogenesis of senile (type II) osteoporosis (Avioli *et al.* 1965; Gallagher *et al.* 1979). In particular, the age-related decrease in the circulating level of $1,25(OH)_2D_3$ results in decreased vitamin D-dependent intestinal Ca absorption (Avioli *et al.* 1965; Gallagher *et al.* 1979), negative Ca balance, secondary hyperparathyroidism and bone loss (Malm *et al.* 1955; Armbrecht *et al.* 1980, 1982; Gray & Gambert, 1982; Tsai *et al.* 1984; Wong *et al.* 1997, 2000).

The biosynthesis of $1,25(OH)_2D_3$ is catalysed by 25-hydroxyvitamin D 1-α hydroxylase (1-OHase; Henry & Norman, 1974) while the degradation of $1,25(OH)_2D_3$ is initiated by induction of 25-hydroxyvitamin D_3 24-hydroxylase (24-OHase) activity (Akiyoshi-Shibata *et al.* 1994). These enzymes are regulated in a coordinate manner in different physiological conditions to control circulating levels of $1,25(OH)_2D_3$. For example, PTH can increase serum $1,25(OH)_2D_3$ levels by inducing 1-OHase activities and suppressing 24-OHase activities (Armbrecht *et al.* 1982). Similarly, $1,25(OH)_2D_3$ can induce its own breakdown by strongly inducing 24-OHase mRNA expression and activities while suppressing 1-OHase mRNA expression and activities simultaneously (Jones *et al.* 1998). Recently, renal vitamin D receptor (VDR) was also found to be actively involved in regulating renal metabolism of $1,25(OH)_2D_3$ (Beckman & DeLuca, 2002; Healy *et al.* 2003).

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D; 1-OHase, 25-hydroxyvitamin D_3 1-α hydroxylase; 24-OHase, 25-hydroxyvitamin D_3 24-hydroxylase; ALPD, adult rats fed LPD; AND, adult rats fed NPD; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPD, low-phosphate diet; NPD, normal phosphate diet; PTH, parathyroid hormone; VDR, vitamin D receptor; YLPD, young rats fed LPD; YND, young rats fed NPD.

* **Corresponding author:** Dr Man-Sau Wong, fax +852 23649932, email bcmswong@polyu.edu.hk

† These authors contributed equally to the studies.

Down-regulation of renal VDR expression in response to hypocalcaemia can prevent $1,25(OH)_2D_3$-mediated suppression of 1-OHase and induction of 24-OHase during dietary Ca restriction, thereby allowing a net increase in serum $1,25(OH)_2D_3$ levels (Beckman & DeLuca, 2002).

Dietary phosphate restriction is an important regulator of renal 1-OHase and 24-OHase activities and mRNA expression (Wu *et al.* 1996; Tenenhouse *et al.* 2001; Yoshida *et al.* 2001; Zhang *et al.* 2002). The effect of dietary phosphate restriction on vitamin D metabolism appears to be PTH-independent (Tanaka & Deluca, 1975), and dependent upon growth hormone or insulin-like growth factor I (Gray, 1987; Halloran & Spencer, 1988). In young rats, the adaptive response to low-phosphate diet (LPD) results in increased 1-OHase and decreased 24-OHase, leading to increase in serum $1,25(OH)_2D_3$ and intestinal P transport (Hughes *et al.* 1975; Tanaka and Deluca 1975; Lee *et al.* 1979; Wu *et al.* 1996). However, in adult rats, the magnitudes of the increase in serum $1,25(OH)_2D_3$ levels, intestinal P transport as well as net P absorption by LPD are reduced (Tanaka & Deluca, 1975; Wong *et al.* 1997). Previous studies in our laboratory demonstrated that the reduced responses of serum $1,25(OH)_2D_3$ levels to LPD in adult rats (4–6 months of age) was due to their inabilities to increase renal 1-OHase activity during LPD (Wong *et al.* 1997). These results are similar to those observed in studies using older rats (12–24 months of age) (Armbrecht *et al.* 1980, 1982, 1984, 1999, 2003), suggesting that the loss of renal 1-OHase to its stimuli occurs in rats as early as 4–6 months of age.

Our recent study further reported that the age-related changes in renal $1,25(OH)_2D_3$ biosynthesis were due to the altered responses of adult rats to increase renal 1-OHase mRNA as well as protein expression within 5 d of LPD feeding (Lai *et al.* 2003). Since circulating levels of $1,25(OH)_2D_3$ increase in adult rats in the absence of an increase in its biosynthesis during LPD treatment, we hypothesized that the mechanism involved in raising circulating $1,25(OH)_2D_3$ in response to mineral deficiency alters with age. In the present study, we tested the hypothesis by studying the expression of genes involved in renal vitamin D metabolism (renal 1-OHase, 24-OHase and VDR) in young (1-month-old) and adult (6-month-old) rats in response to LPD.

Materials and methods

Animals and diet study

Male Sprague Dawley rats weighing 100–110 g (young, 1 month old) and 462–560 g (adult, 6 months old) were first fed with a normal P diet (NPD; 0·6 % Ca, 0·65 % P; Teklad diet 98 005, Harlan Teklad, Madison, WI, USA) for 2 d and then switched to a low P diet (LPD; 0·6 % Ca, 0·1 % P; Teklad diet 98 004) *ad libitum* for 0–7 d. All diets contained 0.055 μg/g vitamin D_3 and their compositions are summarized in Table 1. Our preliminary experiment indicated that rats fed with either 2 or 10 d of NPD have similar serum Ca, P and renal 1-OHase mRNA expression in both young and adult rats. Thus, 2 d of NPD feeding was chosen as the period of equilibration for all rat groups and rats fed 2 d of NPD (or 0 d of LPD) was chosen as the control group of our study. Rats were placed under ether anaesthesia and exsanguinated via the abdominal aorta. Rats were weighed before and after the diet treatment. Blood was collected for serum chemistry analysis. Serum phosphate and Ca were measured using colorimetric assay kits (Sigma, St. Louis, MO, USA) and serum $1,25(OH)_2D_3$ levels were detected by a competitive assay kit (Immundiagnostik, Bensheim, Germany). The kidneys were removed, immediately frozen in liquid N, and stored at −80 °C until use. In addition, young and adult rats were fed *ad libitum* with NPD or LPD for 10 d to determine the effect of prolonged LPD feeding on renal 1-OHase, 24-OHase and VDR mRNA expressions. All studies were conducted according to the principles and procedures contained in the most recent publication of the *NIH Guide for the Care and Use of Laboratory Animals* National Research Council (1996).

Table 1. Composition of the normal phosphate diet (NPD) and the low-phosphate diet (LPD)

	Composition (g/kg)	
	NPD*	LPD†
Casein	110·0	110·0
Egg-white solid, spray-dried	97·9	97·9
DL-Methionine	3·0	3·0
Sucrose	551·1	575·7
Corn starch	100·0	100·0
Corn oil	50·0	50·0
Cellulose	20·0	20·0
Vitamin mix, Teklad (40 060)	10·0	10·0
Potassium phosphate, monobasic	24·6	–
Calcium carbonate	14·7	14·7
Potassium chloride	5·6	5·6
Sodium bicarbonate	4·6	4·6
Magnesium oxide	3·8	3·8
Sodium chloride	3·7	3·7
Sodium selenite	0·50	0·5
Ferric citrate	0·21	0·21
Manganous carbonate	0·123	0·123
Zinc carbonate	0·056	0·056
Chromium potassium sulphate	0·0193	0·0193
Cupric carbonate	0·011	0·011
Potassium iodate	0·0004	0·0004

*Ca 0.6 %, P 0.65 %: cat. no. 98 005.
†Ca 0.6 %, P 0.1 %; cat. no. 98 004.

RNA isolation and RT-PCR analysis of vitamin D-related genes

The rat kidney was first homogenized with a Polytron. Total RNA was then isolated using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RT-PCR was employed to determine the expression of 1-OHase, 24-OHase and VDR under dietary P restriction. Reverse transcription of mRNA was carried out using 5 μg total RNA in a 20 μl reaction volume containing 0·5 μg oligo dT primer (Life Technologies), 200 U™ RT (Life Technologies), 0·5 mM-dNTP, 10 mM-dithiothreitol, in the reaction buffer of 50 mM-Tris-HCl (pH 8·3), 75 mM-KCl, 3 mM-$MgCl_2$. Total RNA was initially denatured at 70°C for 10 min and immediately chilled on ice. First-strand cDNA was synthesized after 50 min at 42°C, followed by 70°C for 15 min to inactivate the RT. Two units of Rnase H were then added followed by incubation at 37°C for another 20 min.

The optimal PCR cycles for each gene product were determined to ensure the PCR products were obtained within the linear logarithmic phase of each amplification curve. Gene-specific primers used in the RT-PCR reaction of each gene are listed in Table 2. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control for mRNA normalization. The PCR condition is as follows 20 μl: the 20 μl cDNA reaction mixture was added to 100 μl PCR mixture containing 0·1 μM-specific primers, 1× PCR reaction buffer, 200 μM-dNTP and 2·6 U DNA polymerase. Samples were first denatured at 95°C for 3 min, amplified for optimized cycles and finally extended at 72°C for 5 min. Each cycle consisted of 95°C for 1 min, 53°C for 30 s and 72°C for 3 min. The PCR products were analysed by agarose gel electrophoresis. The intensity of the PCR products was quantified by Lumi-Image™ F1 Workstation with the LumiAnalyst version 3.1 software (Roche Molecular Biochemicals, Mannheim, Germany). The band intensity captured from the Lumi-Image F1 Workstation was within the saturation limit. Gene expression was expressed as a ratio of the mRNA level of the enzyme component of interest to that of GAPDH.

To ensure conditions for RT-PCR can be used for semi-quantitative comparison of gene expression between different samples, cDNA template derived from the reverse transcription was serially diluted and amplified with the specified condition for each individual gene to test the linearity of the intensity of the PCR product. The linearity of the RT-PCR analysis was confirmed when the amount of the input cDNA was proportional to the signal intensity of the corresponding gene. All PCR products were sequence-verified to ensure the product representing the desired gene product. The PCR product bands were excised from the agarose gel and then purified using the gel extraction kit. The nucleotide sequences of the PCR products were determined by the dideoxynucleotide chain termination kit (Perkin Elmer, Foster, CA, USA) according to the manufacturer's protocol. The sequence read from the ABI 310 sequencer (Perkin Elmer) was subjected to the Blast search.

Statistical analysis

Data are reported as means with their standard errors. For the time-course experiment, significance of differences among group means were determined using one-way ANOVA followed by Tukey's method by GraphPad Prism version 4.02 (GraphPad Prism, San Diego, CA, USA) on serum Ca, phosphate, $1,25(OH)_2D_3$, 1-OHase and 24-OHase mRNA expression levels. Group means of rats with different duration of LPD treatment were compared within their age group. Two-way ANOVA was also used to determine the interaction between diet treatment and age for both the time-course experiment as well as for the prolonged LPD treatment experiment on the serum Ca, phosphate, $1,25(OH)_2D_3$, 1-OHase, 24-OHase and VDR mRNA expression levels. Group means of young rats fed NPD (YND) or LPD (YLPD) and adult rats fed NPD (AND) or LPD (ALPD) were compared. Student's *t* test was used to compare the means of percentage change between young and adult rats in 1-OHase, 24-OHase and VDR mRNA expression. Group means differing by *P* values of 0·05 and less are considered statistically significant.

Results

The effects of dietary P restriction on weight gain, serum P and Ca in young and adult rats are summarized in Table 3. Upon 1 d of LPD treatment, serum P decreased to 7·3 (SEM 0·1) mg/dl ($P<0·01$), *v.* 11·8 (SEM 0·5) mg/dl day 0 and remained low throughout the duration of LPD treatment ($P<0·001$) in young rats. In contrast, serum Ca was not significantly increased in young rats on day 1 of LPD treatment from 9·9 (SEM 0·2) mg/dl for day 0 to 10·6 (SEM 0·4) mg/dl for day 1) and remained unchanged for the duration of the study. In adult rats, serum P was reduced to 3·2 (SEM 0·6) mg/dl ($P<0·001$) *v.* 6·3 (SEM 0·3) mg/dl for day 0 upon 1 d of LPD treatment and remained low throughout the duration of LPD treatment. Serum Ca was unchanged in adult rats during the course of LPD treatment (NS, *v.* 10·6 (0·6) mg/dl for day 0).

The responses of serum $1,25(OH)_2D_3$ level to LPD treatment in both young and adult rats are shown in Fig. 1. In young rats, serum $1,25(OH)_2D_3$ level was increased significantly to 5-fold of the basal level by day 3 of LPD treatment and remained elevated until day 7 of LPD treatment ($P<0·01$, *v.* day 0). This elevation was also observed in adult rats fed 3–7 d of LPD ($P<0·05$, *v.* day 0); however, the extent of up-regulation was lower than that of the young rats (2–3-fold in adult rats *v.* 4–5-fold in young rats). In two-way ANOVA analysis, there is no interaction between diet treatment and age on the mean serum P and Ca concentrations of rats (serum P NS, F 1·65, serum Ca NS, F 0·14). However, significant interactions were observed between the effect of diet and age on the serum $1,25(OH)_2D_3$ level ($P=0·0011$, F 5·92).

The effects of LPD treatment on renal 1-OHase and 24-OHase mRNA expressions in young rats were determined by using RT-PCR as shown in Fig. 2. Renal 1-OHase mRNA abundance in young rats began to increase on day 1 of LPD treatment ($P<0·01$, *v.* day 0), reached 3-fold by day 3 of LPD treatment

Table 2. Primers used for vitamin D-related genes

Gene	Sequence	Accession no.
1-OHase	Forward primer: AAGGCAGCTGTCATCATCTCTC Reverse primer: GATAGACCATGGCAGGAGATAG	AB001992
24-OHase	Forward primer: CAGTATTCCTAAGACGGCGAAC Reverse primer: TCTATCTCCACACCCTGAAACC	X59506
VDR	Forward primer: AACACGCTGCAGACCTACATCC Reverse primer: GGAGTGAATGCGTCTCTTCTGC	J04147
GAPDH	Forward primer: TGGCATCGTGGAAGGGCTCATG Reverse primer: CCACCACCCTGTTGCTGTAGCC	X00972

1-OHase, 25-hydroxyvitamin D_3 1-α hydroxylase; 24-OHase, 25-hydroxyvitamin D_3 24-hydroxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VDR, vitamin D receptor.

Table 3. Weight gain, serum calcium and phosphate levels in young and adult rats in response to low-phosphate diet (LPD) treatment†

(Mean values with their standard errors of the mean for six rats per group)

Day of LPD treatment	Weight gain/d (g/d)		Serum P level (mg/dl)		Serum Ca level (mg/dl)	
	Mean	SEM	Mean	SEM	Mean	SEM
Young rats						
0	10·3	1·3	11·8	0·5	9·9	0·2
1	9·0	0·6	7·3	0·1*	10·6	0·4
3	6·7	1·7	7·0	0·3**	10·4	0·3
5	5·0	0·5	5·6	0·5**	10·4	0·1
7	6·3	0·4	6·8	0·2**	10·0	0·9
Adult rats						
0	2·0	6·2	6·3	0·3	10·6	0·6
1	4·3	0·9	3·2	0·6**	10·2	0·3
3	1·8	0·8	3·8	0·2**	9·5	0·1
5	4·1	1·6	4·1	0·5**	9·9	0·1
7	−3·8	6·3	4·7	0·3**	9·5	0·1

Mean values were significantly different from those of rats at day 0 within the same age group, $^{**}P<0.01$ and $^{***}P<0.001$.

† Young (1 month old) and adult (6 months old) male Sprague Dawley rats were fed with normal phosphate diet (0·6 % Ca, 0·6 % P) for 2 d and switched to LPD treatment (0·6 % Ca, 0·1 %P) for 0–7 d.

($P<0.001$, *v.* day 0) and gradually returned to basal level by day 7 (Fig. 2(A, B)). The abundance of renal 24-OHase mRNA decreased by more than 90 % upon 1 d of LPD treatment ($P<0.001$, *v.* day 0) and remained suppressed throughout the duration of the study (Fig. 2(A, B)). The results showed that suppression of 24-OHase mRNA occurs more rapidly and to a greater extent than the increase in 1-OHase mRNA. The suppression of renal 24-OHase mRNA was prolonged while the abundance of renal 1-OHase mRNA returned to basal level at 7 d of LPD treatment (Fig. 2(B)).

In contrast to the responses of young rats to LPD, the abundance of renal 1-OHase mRNA did not increase in adult rats throughout the 7 d of LPD treatment (Fig. 3(A, B)). Nevertheless, the abundance of renal 24-OHase mRNA in adult rat decreased by 60 % ($P<0.001$, *v.* 90 % in young rats) upon 3 d of LPD treatment ($P<0.01$, *v.* day 0) and remained suppressed throughout the duration of LPD treatment (Fig. 3(A, B)). In two-way ANOVA analysis, the effects of LPD alone ($P<0.0001$, F 33·42, 1-OHase; $P<0.01$, F 20·43, 24-OHase) and age alone ($P<0.0001$, F 75·71, 1-OHase; $P<0.001$, F 35·58, 24-OHase) on 1-OHase and 24-OHase mRNA expressions were significant in rats. The interaction between LPD treatment and age was significant in mean 1-OHase mRNA expressions ($P<0.001$, F 26·42), but not in mean 24-OHase mRNA expressions ($P>0.5$, F 1·66). The latter suggests that the response of 1-OHase expression, but not 24-OHase expression, to LPD is age-dependent.

To determine if the response of renal VDR mRNA expression to LPD treatment was altered by age, the abundance of VDR mRNA was determined in young and adult rats upon either 0 or 3 d of LPD treatment (Fig. 4). The abundance of renal VDR mRNA decreased upon 3 d of LPD treatment in both young (by 77 %, $P<0.001$, *v.* day 0) and adult (by 51 %, $P<0.05$, *v.* day 0) rats, while the magnitude of changes (% decrease) in response to LPD treatment differed significantly in the two age groups (77 *v.* 51 %, $P<0.001$). In addition, our results indicate that the basal level of renal VDR mRNA in adult rats was significantly lower than that of the young rats (Fig. 4(B), $P<0.001$).

Our results indicate that adult rats did not increase renal 1-OHase mRNA expression throughout 7 d of LPD treatment. To determine if longer duration of LPD would be needed for the induction of 1-OHase mRNA expression, both young and adult

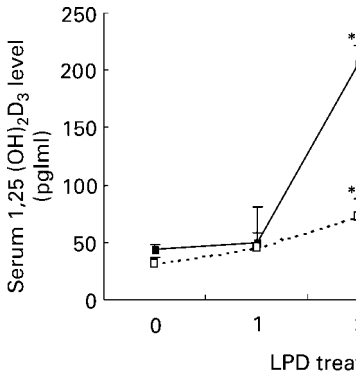

Fig. 1. Serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) level in young and adult rats during low-phosphate diet (LPD) treatment. Both young (▪) and adult (□) rats showed a significant increase in serum $1,25(OH)_2D_3$ level by 3 d of LPD treatment. The level remained elevated till day 7. Mean values were significantly different from those of rats at day 0 within the same age group, $^{*}P<0.05$ and $^{**}P<0.01$, with four rats per group.

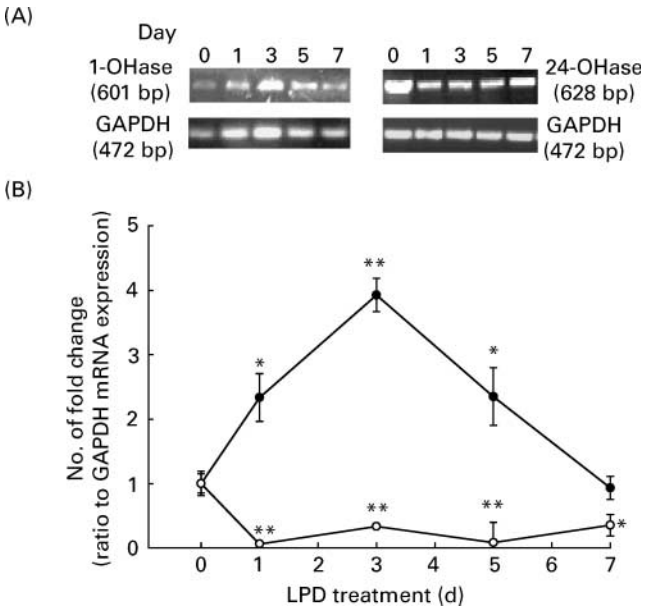

Fig. 2. Renal 25-hydroxyvitamin D_3 24-hydroxylase (24-OHase) and 25-hydroxyvitamin D_3 1-α hydroxylase (1-OHase) mRNA expression in young rats in response to low-phosphate diet (LPD). Young male Sprague Dawley rats (1 month old) were fed with normal phosphate diet (0·6 % Ca, 0·6 % P) for 2 d and switched to LPD (0·6 % Ca, 0·1 % P) for 0–7 d. Total RNA was extracted from kidney, and the conditions for RT-PCR are described on p. 300. (A), Time course of renal 1-OHase and 24-OHase mRNA in response to LPD treatment. (B), Graphical presentation of the coordinate expression of renal 1-OHase mRNA (•) and 24-OHase mRNA (o) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The expression of 1-OHase mRNA reached a maximum by day 3 significantly, but returned to the basal level by day 7 of LPD treatment. LPD treatment decreased the expression of 24-OHase mRNA significantly by day 1 and the effect was prolonged throughout the course of treatment. Data are expressed as means with their standard errors from three to four rats per group. For each group, statistical analysis was performed using one-way ANOVA followed by the use of Tukey's test as post-test analysis. Mean values were significantly different from those of rats at day 0 within the same age group, $^{**}P<0.01$ and $^{***}P<0.001$.

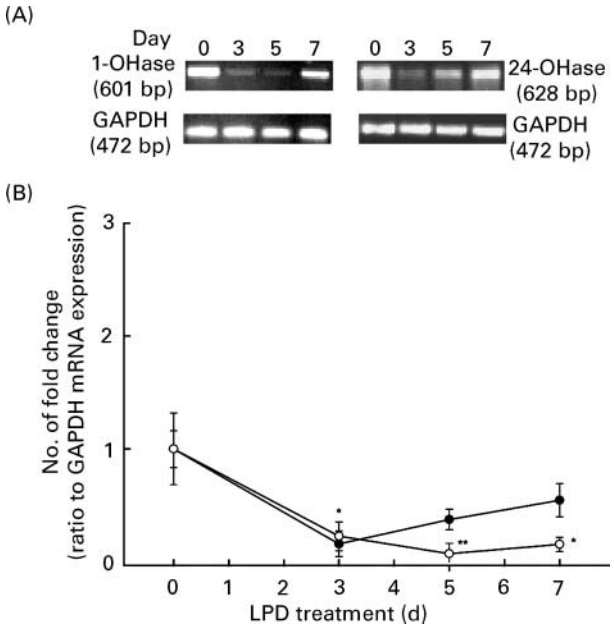

Fig. 3. Renal 25-hydroxyvitamin D_3 24-hydroxylase (24-OHase) and 25-hydroxyvitamin D_3 1-α hydroxylase (1-OHase) mRNA expression in adult rats in response to low-phosphate diet (LPD). Adult male Sprague Dawley rats (6 months old) were fed with normal phosphate diet (0·6 % Ca, 0·6 % P) for 2 d and switched to LPD (0·6 % Ca, 0·1 % P) for 0–7 d. Total RNA was extracted from kidney and the conditions for RT-PCR are described in p. 300. (A), Time course of renal 1-OHase and 24-OHase mRNA on response to LPD treatment. (B), Graphical presentation of the coordinate expression of renal 1-OHase (•) and 24-OHase (o) mRNA relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The mRNA expression of both enzymes was down-regulated by day 3 and their expressions remained low untill day 7. Data are expressed as means with their standard errors from three to four rats per group. For each rat group, statistical analysis was performed using one-way ANOVA followed by the use of Tukey's test as post-test analysis. Mean values were significantly different from those of rats at day 0 within the same age group, $^{**}P<0{\cdot}01$ and $^{**}P<0{\cdot}001$.

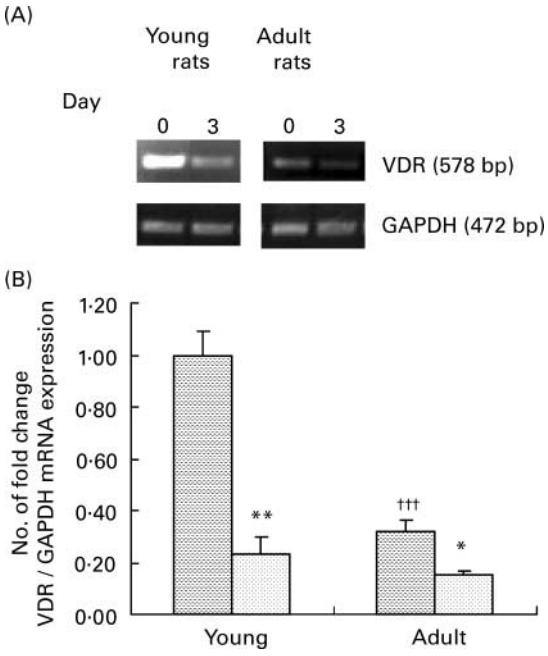

Fig. 4. Renal vitamin D receptor (VDR) expression in response to 3 d of low-phosphate diet (LPD) treatment in young and adult rats. Young (1 month old) and adult (6 months old) male Sprague Dawley rats were fed with normal phosphate diet (NPD, 0·6 % Ca, 0·6 % P) or LPD (0·6 % Ca, 0·1 % P) for 3 d. Total RNA was extracted from kidney and the conditions for RT-PCR are described on p. 300. (A), RT-PCR analysis of VDR mRNA expression in response to 3 d of LPD treatment in young and adult rats. (B), Graphical representation indicating the change of mRNA expression of VDR. Both age groups showed a significant decrease in VDR mRNA expression in response to LPD treatment. Basal expression of VDR was found to be higher in the younger group. Values represent means with their standard erros of three to four rats per group. Expression of VDR mRNA was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Mean values were significantly different from those of rats fed with NPD within the same age group, $^{*}P<0{\cdot}05$ and $^{**}P<0{\cdot}001$. Mean value was significantly different from that of young rats fed with NPD, $\dagger\dagger\dagger P<0{\cdot}001$. (■) NPD; (□) LPD3

rats were fed with either NPD or LPD for 10 d and the expression levels of 1-OHase, 24-OHase and VDR mRNA were determined. The effects of diet treatment on weight change, serum Ca, P and $1,25(OH)_2D_3$ are summarized in Table 4. Serum P level decreased upon 10 d of LPD treatment in both age groups (YND *v.* YLPD10, $P<0{\cdot}01$; AND *v.* ALPD10, $P<0{\cdot}01$). The basal level of serum P was lower in adult rats (YND *v.* AND, $P<0{\cdot}001$). LPD treatment for 10d increased serum Ca level significantly ($P<0{\cdot}001$, *v.* YND) in young rats but not in adult rats. Both age groups showed an elevation of serum $1,25(OH)_2D_3$ level in response to LPD treatment (YND *v.* YLPD10, $P<0{\cdot}001$; AND *v.* ALPD10, $P<0{\cdot}001$) (Table 4). As shown in Fig. 5, 10 d of LPD treatment increased renal 1-OHase mRNA expression in young (YND *v.* YLPD10, $P<0{\cdot}001$) and adult rats (AND *v.* ALPD10, $P<0{\cdot}05$), the magnitude of up-regulation in young rats was higher than that in adult rats (125 *v.* 80 %, $P<0{\cdot}05$). However, prolonged LPD treatment suppressed renal 24-OHase mRNA expression significantly in adult rats (AND *v.* ALPD10, $P<0{\cdot}001$) but not in young rats (YND *v.* YLPD10); while the suppression of renal VDR mRNA expression by LPD treatment was significant only in young rats (YND *v.* YLPD10, $P<0{\cdot}05$) but not in adult rats (AND *v.* ALPD10). In two-way ANOVA analysis, significant interactions between the effects of LPD and age were found for both renal 1-OHase and 24-OHase mRNA expressions, but not for renal VDR mRNA expression (1-OHase, $P<0{\cdot}05$, F 4·97; 24-OHase, $P<0{\cdot}05$, F 4·95; VDR, F 0·22).

Discussion

The increase in renal $1,25(OH)_2D_3$ production during dietary Ca or phosphate restriction mediates vitamin D-dependent intestinal Ca and phosphate absorption. However, regulation of vitamin D metabolism changes with age (Armbrecht *et al.* 1980, 1982; Gray & Gambert, 1982; Tsai *et al.* 1984; Friedlander *et al.* 1994; Wong *et al.* 1997, 2000). In older rats (12 months old), the ability to increase renal $1,25(OH)_2D_3$ production and 1-OHase mRNA expression in response to low-Ca diet feeding was reduced (Armbrecht *et al.* 1980, 1982, 1984, 2003). Previous studies in our laboratory demonstrated that the abilities to increase renal $1,25(OH)_2D_3$ production in response to PTH (Friedlander *et al.* 1994), LPD (Wong *et al.* 1997) or low-Ca diet (Wong *et al.* 2000) were blunted in adult rats (4–6 months old). Our recent studies further reported that the lack of an increase in renal 1-OHase activities during 5 d of LPD was due to the inability of adult rats to increase its mRNA expression (Lai *et al.* 2003). In those studies, serum $1,25(OH)_2$ D_3 levels increased in response to a low Ca diet and LPD in adult rats

without the increase in activities or mRNA expression of renal 1-OHase, suggesting that other mechanisms are involved in raising serum $1,25(OH)_2D_3$ levels.

The results of the present study support our hypothesis that the mechanisms involved in raising serum $1,25(OH)_2D_3$ levels during LPD treatment alter with age. Our results clearly showed that young rats increased serum $1,25(OH)_2D_3$ levels by increasing its biosynthesis via the induction of renal 1-OHase mRNA expression (Fig. 2) while decreasing its metabolic clearance via the suppression of renal 24-OHase (Fig. 2) and VDR (Fig. 4) mRNA expression during short-term LPD treatment. In contrast, adult rats increased serum $1,25(OH)_2D_3$ levels by decreasing its metabolic clearance via the down-regulation of 24-OHase (Fig. 3) and VDR (Fig. 4) mRNA expression during short-term LPD treatment, while the induction of 1-OHase mRNA expression in adult rats only occurred upon a prolonged period of LPD treatment (Fig. 5).

The results of the present study suggest that down-regulation of metabolic clearance by 24-OHase is also an important mechanism for the up-regulation of circulating $1,25(OH)_2D_3$ during LPD. As shown in Fig. 1, serum $1,25(OH)_2D_3$ increased in response to LPD during which renal 1-OHase mRNA was not up-regulated in both young (day 7 of LPD treatment, Fig. 2) and adult rats (days 3–7 of LPD treatment, Fig. 3). Our previous study demonstrated that LPD increased 1-OHase mRNA and protein in young rats, but not adult rats, in a time-dependent manner in which an increase in renal 1-OHase mRNA on day 3 of LPD treatment was followed by an increase in renal 1-OHase protein on days 3 and 5 of LPD treatment (Lai *et al.* 2003). In that particular study, we have shown that an increase in protein stability of 1-OHase is also a mechanism involved in up-regulation of the enzyme expression in young rats during LPD treatment (Lai *et al.* 2003). In the present study, the result clearly showed that the suppression of renal 24-OHase mRNA expression occurred on day 1 of LPD treatment and remained suppressed throughout the duration of LPD treatment in both young and adult rats. Thus, an increase in protein stability of renal 1-OHase protein as well as a decrease in metabolic clearance by 24-OHase might contribute to the discrepancies between observed serum $1,25(OH)_2D_3$ as well as renal 1-OHase mRNA in young rats fed LPD; while the suppression of metabolic clearance by 24-OHase would likely be the major mechanism involved in up-regulation of serum $1,25(OH)_2D_3$ in adult rats during LPD.

The results of our study also agree with the studies by Zhang *et al.* (2002) that the response of renal 24-OHase mRNA suppression to LPD precedes that of renal 1-OHase mRNA up-regulation. In their studies, they raised the possibility that a decrease in renal catabolism of $1,25(OH)_2D_3$ together with an increase in its renal synthesis contribute to the increase in serum $1,25(OH)_2D_3$ levels during phosphate restriction in young mice. Furthermore, the present study showed that basal 24-OHase mRNA expression increased with age (Fig. 5) and that the degree of suppression of 24-OHase mRNA during short-term LPD treatment decreased with age (90 *v.* 60 %). Thus, in addition to the age-related reduction in 1-OHase responses, these results showed that metabolic clearance of $1,25(OH)_2D_3$ by 24-OHase also increased with age. The latter provides another explanation for why adult animals have a much reduced response of serum $1,25(OH)_2D_3$ to phosphate deficiency (Fig. 1).

A recent study showed that down-regulation of renal VDR expression was a mechanism involved in enhancement of renal 1-OHase mRNA expression as well as suppression of renal 24-OHase mRNA expression. The decrease in functional VDR can reduce the inhibition of 1-OHase mRNA (Murayama *et al.* 1999) as well as the stimulation of 24-OHase mRNA (Roy & Tenenhouse, 1996) normally induced by $1,25(OH)_2D_3$. The present study showed that renal VDR mRNA abundance decreased in response to LPD treatment in both young and adult rats. Such reduction of VDR expression might facilitate the increase in 1-OHase mRNA expression and the decrease in 24-OHase mRNA expression in young rats during LPD treatment. The fact that LPD treatment could down-regulate renal VDR mRNA expression in adult rats, however, suggests that the inability of adult rats to increase renal 1-OHase mRNA expression by 3 d of LPD treatment is unlikely to be due to an age-related alteration of VDR regulation.

To determine if longer duration is required for the up-regulation of renal 1-OHase by a diet of 0·1 % P in adult rats, we extended the duration of LPD treatment from 7 to 10 d. Our results clearly showed that renal 1-OHase mRNA expression could be induced by 10 d of LPD treatment in adult rats; however, the mechanism involved in its up-regulation by LPD might be different from their younger counterparts. In young rats, renal 1-OHase mRNA was up-regulated

Table 4. Weight gain, serum calcium, phosphate and 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) levels in young and adult rats in response to 10 d of low-phosphate diet (LPD) treatment‡

(Mean values and their standard errors of the mean of eight rats per group)

	Weight change/d (g/d)		Serum P level (mg/dl)		Serum Ca level (mg/dl)		Serum $1{\cdot}25(OH)_2D_3$ level (pg/ml)	
Diet treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Young rats								
YND	6·66	0·89	11·88	0·42	9·70	0·19	52·82	6·86
YLPD	5·80	0·17	6·37	0·25*	11·64	0·46**	209·63	15·71**
Adult rats								
AND	4·16	1·18	6·31	0·09*	10·36	0·25	29·05	1·39*
ALPD	4·75	0·41**	4·23	0·13*†††	10·73	0·06	90·99	7·16**†††

Mean values with were significantly different from those of YND group, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$, Mean values were significantly different from those of AND group, †††$P<0{\cdot}001$.

‡Young (1 month old, YND and YLPD) and adult (6 months old, AND and ALPD) male Sprague Dawley rats were fed with either normal phosphate diet (NPD, 0·6 % Ca, 0·6 % P) or LPD (0·6 % Ca, 0·1 %P) for 10 d, respectively.

during which serum P was reduced by LPD treatment (Table 3); whereas, in the case of adult rats, the induction did not occur at an earlier time even when serum P was reduced. Hypophosphataemia alone is not sufficient for the induction of renal 1-OHase mRNA in adult rats. It is possible that the decrease in serum P *per se* is not the primary signal for triggering the up-regulation of renal 1-OHase mRNA expression in both young and adult animals and that other signals that result from the secondary responses to hypophosphataemia are responsible for the 1-OHase regulation during LPD. In the case of adult rats, it is likely that a lag time is needed for generating the signals in response to hypophosphataemia to induce up-regulation of renal 1-OHase mRNA during LPD treatment. Further studies will be needed to identify the mechanism involved in age-related alteration of renal 1-OHase mRNA expression during LPD treatment.

The mechanism whereby LPD treatment regulates vitamin D metabolism is poorly understood. It appears to be PTH-independent (Tanaka & Deluca, 1975), and dependent upon growth hormone or insulin-like growth factor I (Gray, 1987; Halloran & Spencer, 1988). Recently, two novel genes, PHEX and FGF-23, have been discovered to be involved in regulation of phosphate homeostasis (Takeda *et al.* 2004). The PHEX gene encodes an endopeptidase that breaks down FGF-23; while FGF-23 acts as a potent regulator to down-regulate renal phosphate transport and circulating $1,25(OH)_2D_3$ levels (Shimada *et al.* 2004). The PHEX gene is mutated in X-linked hypophosphataemic rickets while a mutated form of FGF-23 is found to be accumulating in patients with autosomal dominant hypophosphataemic rickets. In both cases, the accumulation of FGF-23 leads to excessive P wasting and abnormal vitamin D metabolism. It will be of interest to determine if the expression of FGF-23 and PHEX alter with age and whether the age-related alteration in vitamin D metabolism is associated with changes in FGF-23 or PHEX expression.

In summary, the results in the present study support our hypothesis that the mechanism involved in increasing circulating $1,25(OH)_2D_3$ levels alters with age. In young rats, LPD treatment induced renal 1-OHase mRNA expression, and decreased renal 24-OHase and VDR mRNA expression, which results in a rapid increase in serum $1,25(OH)_2D_3$ levels to meet the high demand for mineral for skeletal growth; whereas, in adult rats, LPD treatment initially increased serum $1,25(OH)_2D_3$ levels by suppression of renal 24-OHase and VDR mRNA expression, followed by a delayed induction of renal 1-OHase mRNA expression. The overall decrease in responses of vitamin D metabolism in adult rats to LPD treatment might be part of a post-maturational process that signifies the decrease in skeletal requirement for mineral with age.

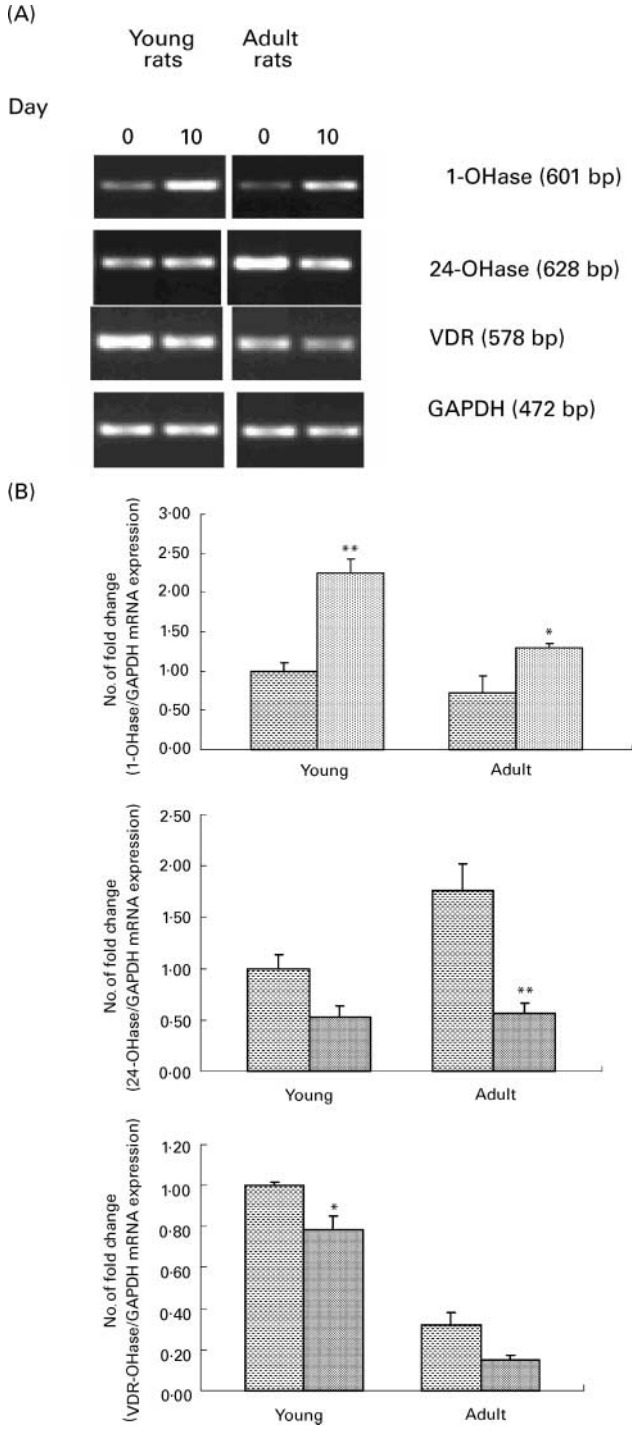

Fig. 5. Renal 25-hydroxyvitamin D_3 1-α hydroxylase (1-OHase), 25-hydroxyvitamin D_3 24-hydroxylase (24-OHase) and vitamin D receptor (VDR) mRNA expressions in response to 10 d of low-phosphate diet (LPD) treatment in young and adult rats. Young (1 month old) and adult (6 months old) male Sprague Dawley rats were fed with either normal phosphate diet (NPD, 0·6 % Ca, 0·6 % P) or LPD (0·6 % Ca, 0·1 % P) for 10 d (LPD10). (A), RT-PCR analysis of 1-OHase, 24-OHase and VDR mRNA expression in response to 10 d of LPD treatment in young and adult rats. (B), Renal expression of 1-OHase (top panel), 24-OHase (middle panel) and VDR (bottom panel) mRNA upon 10 d of LPD treatment. LPD treatment for 10 d significantly increased 1-OHase mRNA expression in both young and adult rats. 24-OHase mRNA expression was significantly suppressed in adult rats in response to prolonged LPD treatment. Renal VDR mRNA expression was found in both age groups. However, significant suppression was only found in young rats in response to prolonged LPD treatment. Values represented means with their standard errors of eight animals per group and were analysed by two-way ANOVA. Expression of mRNA was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Mean values were found to be significantly different from those of rats fed NPD within the same age group, $^*P<0{\cdot}05$ and $^{**}P<0{\cdot}001$, (▨) NPD; (▩) LPD 10.

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