



Effect of hyperphosphatemia on gene expression of the Na-Pi cotransporter in rats

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ABSTRACT. We investigated the effect of high phosphorus content on the sodium-phosphate cotransporter (NaPi-IIa and NaPi-III). Forty-eight Sprague-Dawley rats were divided into 3 groups: high-phosphorus group (HP) with fructose diphosphate sodium injection; self-manufactured low-phosphorus diet group (LP); and normal diet group (NP). At the 1st, 2nd, 4th, and 6th weeks, 4 rats from each group were sacrificed for detecting serum levels of calcium, phosphorus, and intact parathyroid hormone. Semi-quantitative retrovirus-polymerase chain reaction was used to detect the expression of NaPi-IIa and NaPi-III mRNA in kidney. At the 1st, 2nd, 4th, and 6th weeks, serum phosphorus and parathyroid hormone levels in HP group were significantly higher than those in LP and NP groups ($P < 0.05$). Serum calcium levels in the 3 groups showed no difference ($P > 0.05$). Comparing the expression of NaPi-IIa mRNA in HP group with LP and NP groups, NaPi-IIa mRNA expression was significantly reduced in HP group ($P < 0.05$), while NaPi-IIa mRNA expression in LP group began

increasing at the 4th week ($P < 0.05$). At the 1st, 2nd, and 4th weeks, the expression of NaPi-III mRNA in HP, LP, and NP groups showed no clear differences ($P > 0.05$), while at the 6th week in HP group, NaPi-III mRNA expression was slightly increased compared to in LP and NP groups ($P < 0.05$). Hyperphosphatemia significantly affected NaPi-IIa and NaPi-III mRNA expression, and a factor promote an increase in intact parathyroid hormone independently of calcium.

Key words: Hyperphosphatemia; Kidney; Sodium-phosphate cotransporter

INTRODUCTION

Recent studies have demonstrated that hyperphosphatemia is closely related to chronic renal failure, secondary hyperthyroidism, and chronic renal failure-related cardio-cerebrovascular diseases. Metabolism of phosphorus occurs mainly through the kidneys and digestive tract. Under normal conditions, phosphorus excreted through the kidney accounts for approximately 70% of the total excretion, and approximately 30% is excreted in the feces (Goodman, 2004; Yuan and Hand Du, 2010). Phosphorus intake and excretion are nearly equal in normal adults, so the gastrointestinal system and kidneys maintain the balance of body phosphorus metabolism. Numerous studies have confirmed that the sodium-dependent phosphate cotransporter includes a group of Na⁺-dependent transmembrane proteins, which are located in the cell membrane. These transporters play a major role in phosphate metabolism balance. Three types of sodium-phosphate cotransporter have been isolated from mammalian cell membranes: type I sodium-dependent phosphate cotransporter (NaPi-I), type II sodium-dependent phosphate cotransporter (NaPi-II), and type II sodium-dependent phosphate cotransporter (NaPi-III); there are 3 types of NaPi-II: type IIa (NaPi-IIa), type IIb (NaPi-IIb), and type IIc (NaPi-IIc) (Jin et al., 2010). Therefore, the goal of present study was to elucidate the correlation between hyperphosphatemia and the Na-Pi cotransporter, as well as to provide an experimental basis for the clinical treatment and research of hyperphosphatemia and related complications.

MATERIAL AND METHODS

Materials

Animals and grouping

Forty-eight 6-8-week-old Sprague-Dawley rats, weighing 190-200 g, were used in this study. The rats were provided by the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University. The animals were then divided randomly into 3 groups: 1) high serum phosphorus group (HP); 2) low serum phosphorus group (LP); and 3) normal serum phosphorus group (NP). Rats were individually maintained under the same conditions. The distribution of the phosphorus diets was as follows: HP group: 100 mg/kg fructose diphosphate was administered to the rats every day by intraperitoneal injection; LP group: diet containing 0.2% phosphorus and 0.5% calcium according to AIN-93G standard; NP group: diet containing approximately 0.6-1.2% phosphorus (Reeces et al., 1993; Mao et al., 2006). Four animals were sacrificed at the end of the 1st, 2nd, 4th, and 6th week of fixation, and then blood was drawn from the abdominal artery, and the renal cortex was obtained.

Methods

Detection of serum phosphorus, calcium, and intact parathyroid hormone (iPTH)

Serum levels of phosphorus, calcium, and iPTH was determined using a 2-point method on a Hitachi 7600 automatic biochemical analyzer (Tokyo, Japan) (cat. DSL-8000, Beckman Coulter, Inc. USA).

Detection of NaPi-IIa-mRNA and NaPi-III-mRNA from kidney

The renal cortex and medulla were separated and a 50-mg sample was taken from the cortex. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the purity and content of RNA was measured using a Ultrospec 2000 photoelectric colorimeter (GE Life Sciences, Little Chalfont, UK). The A260/280 of the extracted RNA was required to be the range of 1.8-2.0. cDNA was synthesized using reverse transcription kits (Applied Biosystems, Foster City, CA, USA) with glyceraldehyde 3-phosphate dehydrogenase as an internal reference. Primer sequences were designed according to those reported in previous studies (Custer et al., 1994; Zeng et al., 2005). NaPi-IIa, GAPDH and NaPi-III primers were synthesized by Shanghai Biological Technology Service Company (Shanghai, China). The polymerase chain reactions were initiated by denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, followed by renaturation at 55°C for 60 s, elongation at 72°C for 90 s, and elongation at 72°C for 5 min for 35 cycles. Polymerase chain reaction products were separated by 1% agarose gel electrophoresis and the results were photographed under UV light. The absorptiometry of each strip was obtained using a GIS gel image analysis system (GI52010, catalog number 70247-1; Tianmeng Inc., Xuzhou, China) and comparison of the relative expression levels of this gene; each experiment was conducted more than 3 time (Table 1).

Table 1. Polymerase chain reaction primers.

Target gene	Upstream primer (5'-3')	Downstream primer (5'-3')	Amplified products (bp)
GAPDH	AGATCCACAACGGATACATT	TCCCTCAAGATTCACAGCAA	309
NaPi-IIa	CATGGCCAAGGCACTGGGCAAA	TAGAGCCGGGTGGCATTGTGGTG	323
NaPi-III	CATCTCGGTGGATGTGC	TGTTGGTCTCCTCCTCA	306

Statistical analysis

Statistical analysis was performed using SPSS13.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate statistical significance. Data are expressed as the means \pm SD, and analysis of the 3 groups was performed using one-way analysis of variance.

RESULTS

Effects of different diets

Detection results

LP contained 0.19% phosphorus, while NP contained 0.73% phosphorus, which was

consistent with the expected standard. Animals survived until the end of the experiment, except 1 rat in the HP group that died at the 6th week.

Detection of serum biochemical parameters in rats

Dynamic observations of the serum phosphorus, calcium, and iPTH levels are shown in Table 2. The results revealed that serum phosphorus and iPTH levels in the HP group were significantly higher than those in the LP and NP groups ($P < 0.05$). iPTH was higher at the 2nd week than at the 1st week, and secretion increased gradually in the HP group ($P < 0.05$). There was no significant difference in serum calcium among the HP, LP, and NP groups ($P > 0.05$). Serum phosphorus levels in the LP group was lower than those in the NP from the 2nd week ($P < 0.05$), and serum iPTH and phosphorus levels decreased significantly at the 6th week ($P < 0.05$).

Table 2. Biochemical index of rats (means \pm SD).

Group	NP	LP	HP
1 week after postoperative			
P (mM)	2.84 \pm 0.64	2.78 \pm 0.59	4.04 \pm 0.65*
Ca (mM)	1.23 \pm 0.11 ^a	1.22 \pm 0.09	1.20 \pm 0.06
iPTH (pg/mL)	31.94 \pm 6.93	31.65 \pm 17.52	67.69 \pm 31.42*
2 weeks after postoperative			
P (mM)	3.17 \pm 0.48 ^v	2.40 \pm 0.32	4.10 \pm 0.59*
Ca (mM)	1.22 \pm 0.09 ^a	1.29 \pm 0.08	1.21 \pm 0.08
iPTH (pg/mL)	33.72 \pm 10.19	33.07 \pm 12.73	75.33 \pm 17.25**
4 weeks after postoperative			
P (mM)	3.05 \pm 0.56 ^v	2.12 \pm 0.84	3.97 \pm 0.27 [†]
Ca (mM)	1.19 \pm 0.07 ^a	1.20 \pm 0.07	1.21 \pm 0.07
iPTH (pg/mL)	32.40 \pm 5.03	30.55 \pm 5.41	80.45 \pm 22.76**
6 weeks after postoperative			
P (mM)	2.79 \pm 0.6 ^{v9}	2.05 \pm 0.79	4.27 \pm 0.68 [†]
Ca (mM)	1.21 \pm 0.06 ^a	1.14 \pm 0.11	1.22 \pm 0.14
iPTH (pg/mL)	31.98 \pm 7.86 ^v	28.02 \pm 11.79	85 \pm 17.62**

*HP compared with LP and NP in serum P and iPTH at the same time, $P < 0.05$; ^aHP, LP and NP compared with each other in serum Ca, $P > 0.05$; [†]iPTH change compared within the HP, $P < 0.05$; ^vLP compared with NP in serum P and iPTH changes at the same time, $P < 0.05$.

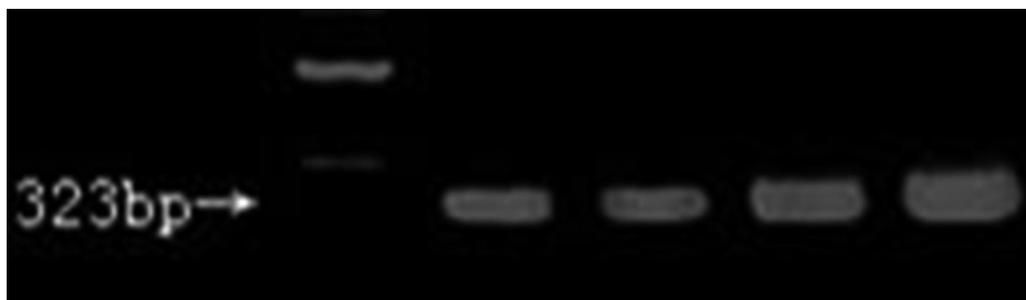
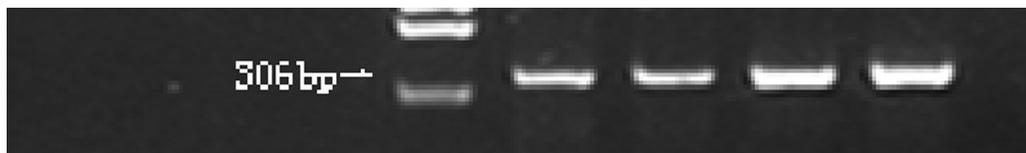
Semi-quantitative analysis of mRNA of NaPi-IIa transporter and NaPi-III transporter in kidneys

The extracted RNA was measured using an Ultrospec 2000 photoelectric colorimeter, and the A260/280 was in the range of 1.8-2.0. The electrophoretic results of RNA showed 5S, 18S, and 28S bands. The extract showed no degradation or contamination. As shown in Table 3, the serum phosphorus level in the HP group increased significantly compared with those in the LP and NP groups ($P < 0.05$); the expression of NaPi-IIa clearly decreased in the kidney ($P < 0.01$), and NaPi-IIa expression decreased over time ($P < 0.05$). Compared with the NP group, NaPi-IIa in the LP group increased significantly ($P < 0.05$). There were no clear difference among the HP, LP, and NP groups in the expression of NaPi-III mRNA at 1, 2, and 4 weeks postoperative ($P > 0.05$), while expression increased in the HP group compared with in the LP and NP groups ($P < 0.05$). NaPi-III mRNA expression did not differ between the LP and NP groups ($P > 0.05$) (Figures 1 and 2).

Table 3. Expression of NaPi-II and NaPi-III mRNA in kidney (means \pm S).

Group	1 week postoperative		2 weeks postoperative		4 weeks postoperative		6 weeks postoperative	
	NaPi-IIa	NaPi-III	NaPi-IIa	NaPi-III	NaPi-IIa	NaPi-III	NaPi-IIa	NaPi-III
NP	3.28 \pm 0.06	2.25 \pm 0.03	3.17 \pm 0.05	2.25 \pm 0.02	3.35 \pm 0.07	2.27 \pm 0.03	3.22 \pm 0.06	2.26 \pm 0.01
LP	2.53 \pm 0.07	2.24 \pm 0.01	2.85 \pm 0.04	2.19 \pm 0.04	3.13 \pm 0.05	2.26 \pm 0.02	3.57 \pm 0.07	2.26 \pm 0.02 ^o
HP	2.25 \pm 0.06 [*]	2.25 \pm 0.02	1.87 \pm 0.07 ^{**}	2.26 \pm 0.01 ^v	1.11 \pm 0.1 ^{2**}	2.28 \pm 0.02 ^v	0.98 \pm 0.09 ^{**}	3.23 \pm 0.03 ^o

Semi-quantitative mRNA means \pm SD were NaPi-IIa mRNA/GAPDH mRNA, NaPi-III mRNA/GAPDH mRNA; ^{*}HP compared with LP and NP in NaPi-IIa mRNA expression at the same time, $P < 0.05$; [#]HP, LP, and NP compared with each other in NaPi-IIa mRNA, $P > 0.05$; ^oHP compared with LP and NP in NaPi-III mRNA expression, $P < 0.05$; ^oNaPi-III mRNA expression was compared between LP and NP, $P > 0.05$; ^vNaPi-III mRNA expression was compared within HP at 1, 2, and 4 weeks, $P > 0.05$.

**Figure 1.** Expression of NaPi-II mRNA in kidney at week 1.**Figure 2.** Expression of NaPi-III mRNA in kidney at week 2.

DISCUSSION

In this experiment, the absorption of calcium was affected by the high phosphorus content in the diet (Zeng et al., 2005). Thus, we adjusted the diet of the HP group, which used a normal diet while injecting fructose diphosphate. The results showed that the iPTH of the rats in HP group increased significantly ($P < 0.05$). In the meanwhile, the rats in HP group were maintained in normal diet and injected high phosphate each day, with appearance of being complicated with secondary hyperthyroidism. HP group rats showed clear hyperphosphatemia in the early stages after 1 week, but biochemical analysis indicated that there were some differences in serum iPTH over time ($P < 0.05$). The HP, LP, and NP groups showed no differences in 1, 2, 4 and 6 week ($P > 0.05$), demonstrating that the hyperphosphatemia promotion of the parathyroid hormone (PTH) was independent of serum calcium levels (Zeng et al., 2004). Pi homeostasis is regulated by PTH, $1\alpha,25$ -dihydroxyvitamin D3, and calcitonin (Miao and Pan, 2007). Therefore, we hypothesized that hyperphosphatemia would lead to an increase in PTH levels and result in secondary hyperparathyroidism (SHPT), causing increased conversion of serum phosphate and decreased

reabsorption of urinary phosphorus. It could relieve the hyperphosphatemia properly and inhibit the further deterioration of SHPT.

Although it was not clear that the expression of renal NaPi-II mRNA decreased, it had been demonstrated that NaPi-IIa is mainly located in the brush apical membrane of proximal tubular cells, which is a major reabsorption transporter of proximal tubular cells. Additionally, NaPi-IIa played an important role. NaPi-IIc also has some significance, and is mainly expressed in the basement membrane of proximal tubular cells. Regulation of NaPi-IIa gene expression mainly occurs on the post-transcriptional level. Cardiovascular activation factors located in the cross-coding region of the NaPi-II mRNA and 3' untranslated region affect the transcriptional activity of cytosolic proteins in the kidney. NaPi-IIa function is regulated by various hormonal and non-hormonal substances (Zhao, 2005). Additionally, we found that the expression of NaPi-IIa mRNA significantly decreased compared with the normal group ($P < 0.05$), as described by Lötscher et al. (1996) and Zeng et al. (2005). However, HP group rats appeared to have hyperphosphatemia and high PTH, and expression of NaPi-IIa mRNA was decreased, indicating that NaPi-IIa expression in the kidney was affected by hyperphosphatemia and high PTH. Reducing the expression of NaPi-IIa mRNA in the renal tubule to decrease the reabsorption of urinary phosphorus may relieve hyperphosphatemia. In our study, we also found that NaPi-IIa transporter mRNA expression increased in the LP group compared to that in the NP group at the 4th week, indicating that the expression and inhibition of NaPi-IIa transporter mRNA was closely correlated with the HP diet and serum PTH, but was not significantly related to serum calcium. Recent studies showed that the expression and function of NaPi-IIa was associated with the gamma-aminobutyric acid receptor-related protein, Na⁺/H⁺ exchange sub-regulatory factor PDZ protein, Na⁺/H⁺ exchanger regulatory factor 1, and Na⁺/H⁺ exchanger regulatory factor 3. Affecting these proteins may reduce or increase the expression of NaPi-IIa (Hernando et al., 2010). The NaPi-III cotransporter, which is involved in transporting phosphorus in parathyroid cells in humans and rats, is important for maintaining intracellular phosphorus concentration under physiological or pathological conditions. It has confirmed that NaPi-III has 2 subtypes: Pit-1 and amphotropic virus receptor. However, only Pit-1 is typically expressed in human smooth muscle cells and the rat parathyroid. Parathyroid cells were found to be the only location of the synthesis of PTH, which plays a key role in NaPi-III. NaPi-III was responsible for the uptake of phosphorus on the membrane, and directly affected PTH synthesis (Wang et al., 2006; Jiang and Wang, 2009). With increased serum phosphate and iPTH, the expression of NaPi-III mRNA increased in the HP group compared with the LP and NP groups at 6 weeks ($P < 0.05$). This revealed that high phosphorus content promoted the secretion of PTH and increased intracellular phosphorus to alleviate the symptoms of hyperphosphatemia by improving the expression of NaPi-III mRNA (Li et al., 2006). The NP group showed no obvious difference compared with the LP group ($P > 0.05$). Renal bone disease, known as renal osteodystrophy, is a common complication in patients with chronic renal failure, including high turnover bone disease (also known as predominant hyperparathyroid bone disease), low turnover bone disease (osteomalacia and adynamic renal bone disease), and mixed bone disease. Yoshioka et al. (2011) found that Slc20a1 (Pit-1) was the member of NaPi-III cotransporter, which play a key role in bone formation and bone mineralization in rats. The expression of NaPi-III mRNA was higher in the HP group than in the LP group, which may be related to bone mineral metabolism. Few studies have examined the function of NaPi-III, so our study lays a foundation for further research of the role and mechanism of the NaPi-III cotransporter in SHPT development. Our results may be useful for developing clinical treatment of SHPT. The regulation and blocking the function of NaPi-III cotransporter should be further examined.

Increasing attention has been given to hyperphosphatemia and its independent disease risk factors. The treatment of hyperphosphatemia will effectively reduce the development of complications, improve patient quality of life, and decrease morbidity and mortality. In this study, we demonstrated that the sodium-phosphate cotransporter mediated the transport of phosphorus under HP concentrations and promoted the occurrence of SHPT. Phosphorus intake occurs mainly through the gastrointestinal tract and is excreted by the kidney. Hyperphosphatemia was prevented by limiting phosphorus intake and promoting the excretion of phosphorus. Therefore, the sodium-phosphate cotransporter should be further examined to develop treatments for hyperphosphatemia.

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